

**ENVIRONMENTAL, GENETIC AND INFLAMMATORY FACTORS
MODIFYING ENDOTHELIAL FUNCTION IN PATIENTS WITH
CORONARY HEART DISEASE**

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ABSTRACT

Background The endothelium plays a vital role in the regulation of blood flow and haemostasis as well as mediating the response to tissue injury and inflammation. Disruption to these pathways is a critical factor in the development of coronary heart disease (CHD) and clinical syndromes such as acute myocardial infarction (MI). Endothelial dysfunction may be demonstrated in patients with CHD and its risk factors such as cigarette smoking with a decrease in the synthesis and release of endothelial-derived factors including the endogenous fibrinolytic factor tissue plasminogen activator (t-PA) and nitric oxide (NO) as well as impaired regulation of vascular tone.

Objectives (1) Although a reduction in endogenous fibrinolytic capacity may be associated with a greater risk of arterial thrombosis, the relationship between acute t-PA release and future atherothrombotic events was unknown. (2) Previous work in healthy subjects had suggested that genetic variation within the t-PA gene may be important in determining inter-individual differences in t-PA release and the risk of future MI; we therefore assessed the effect of t-PA genetic polymorphisms on t-PA release in patients with CHD. (3) Although unable to prove causation, previous studies indicated a relationship between inflammatory markers, plasma t-PA concentrations and the risk of future cardiovascular events. However the direct effect of local arterial inflammation in patients with CHD was unknown. (4) As impaired NO bioavailability may contribute to the development of atherosclerosis, we postulated that prolonging the effect of NO with a highly selective phosphodiesterase type 5 inhibitor (sildenafil citrate) may improve vascular function.

Outcome Measures In a well characterised cohort of patients with stable CHD: atherothrombotic events during follow-up (death from a cardiovascular cause, MI, ischaemic stroke (CVA), hospitalisation for myocardial ischaemia); t-PA genotype; inflammatory cytokines; acute t-PA release and forearm blood flow (FBF) during intra-brachial infusion of vaso-agonists.

Results (1) Net t-PA release was 44% lower in the patients who experienced death, MI, CVA or hospitalisation for myocardial ischaemia ($p \leq 0.02$) over a median follow-up of 34 months. This novel finding validates the application of the forearm model to studying the pathophysiological changes observed in CHD and suggests that the acute endogenous fibrinolytic capacity is important in determining future cardiovascular risk. (2) Despite confirming previous reports of impaired t-PA release and reduced FBF responses in cigarette smokers ($p < 0.05$), we found no effect of a number of t-PA genetic polymorphisms on acute t-PA release. In subjects with established CHD, inter-individual differences in t-PA release appear to predominantly reflect the presence of environment factors such as cigarette smoking. (3) Intra-brachial infusion of tumour necrosis factor- α impaired NO-dependent vasodilatation ($p < 0.001$), increased basal t-PA concentrations and doubled bradykinin-induced t-PA release ($p = 0.006$). This suggests a pathophysiological mechanism whereby circulating levels of inflammatory cytokines are directly related to plasma t-PA concentrations and the risk of future cardiovascular events. Pharmacological interventions with anti-cytokine therapies may have a therapeutic role in subjects at risk of acute coronary syndromes. (4) Despite augmenting the effects of basal NO release and a direct NO donor ($p < 0.05$), a bolus and intravenous infusion of sildenafil did not affect either endothelium-dependent vasodilatation or acute t-PA release. Sildenafil does not modify acute t-PA release and phosphodiesterase type 5 inhibitors are unlikely to reverse the generalised vascular dysfunction seen in patients with coronary heart disease.

To my parents and Anna

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DECLARATION

This thesis represents research undertaken in the Departments of Cardiovascular Research, University of Edinburgh and Cardiology, Royal Infirmary of Edinburgh during the period October 2001 to April 2004. During this time I was working full-time as a British Heart Foundation Junior Research Fellow and I personally undertook all of the vascular studies. In keeping with the nature of this research, assistance with the laboratory assays and vascular studies was gratefully received and has been acknowledged where relevant.

Chapters 4, 5 and 6 have been published in peer reviewed journals. Chapter 3 has been accepted and is awaiting publication. I have copyright permission for inclusion of my published journal manuscripts within this thesis. The thesis has not been accepted in any previous applications for a degree and all sources of information have been acknowledged.

All studies were undertaken in accordance with the regulations of the Lothian Research Ethics Committee and with the Declaration of Helsinki of the World Medical Association. The written informed consent of each subject or patient was obtained before entry into the study.

Simon Robinson

20th October 2006

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CHAPTER 1

INTRODUCTION:

FACTORS MODIFYING ENDOTHELIAL FUNCTION IN PATIENTS WITH CORONARY HEART DISEASE

OVERVIEW

Atherosclerosis is a chronic inflammatory disorder that places a huge global burden of morbidity and mortality on the use of health resources. The clinical syndromes resulting from atherosclerotic conditions such as coronary heart disease are due, in part, to loss of the normal endothelial control of thrombosis and regulation of blood flow. The endogenous fibrinolytic system plays a critical role in preventing unopposed fibrin formation and intravascular thrombosis leading to arterial occlusion, myocardial ischaemia and infarction. Factors regulating fibrinolysis, vasomotor tone and inflammation signalling are thus critical to our understanding of the pathogenesis of coronary heart disease and to assist the development of novel interventional strategies.

1.1 ENDOGENOUS FIBRINOLYSIS

Fibrinolysis describes the hydrolytic cleavage of fibrin by plasmin resulting in the generation of fibrin degradation products secondary to thrombus dissolution. The main fibrinolytic components of plasma are plasminogen, α 2-antiplasmin, tissue plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA). The principal inhibitors of fibrinolysis in the circulation are plasminogen activator inhibitor type-1 (PAI-1) which rapidly inhibits both t-PA and u-PA, and α 2-antiplasmin [Sprengers and Kluft 1987] (Figure 1.1). Under physiological conditions, links between these systems permit localised and timely removal of acutely induced fibrin deposits to ensure blood fluidity while preventing blood loss [Cesarman-Maus and Hajjar 2005]. As well as thrombus dissolution, the fibrinolytic system is also involved in regulating extracellular matrix degradation, cellular migration [Saksela and Rifkin 1988], angiogenesis and tumour invasion [Dano *et al* 1985; Bajou *et al* 1998].

1.1.1 INITIATION OF FIBRINOLYSIS

1.1.1.1 *Plasminogen and Plasmin*

Plasmin, a trypsin-like serine protease is generated from the inactive proenzyme, plasminogen, through the co-ordinated action of a number of enzymes and inhibitors. Plasminogen is a 791 amino acid molecule principally synthesised within the liver comprising a pre-activation sequence, a catalytic protease domain and a number of kringle domains that play a crucial role in the specific recognition of fibrin, cell surfaces, and α 2-antiplasmin [Petersen *et al* 1990]. The conversion of plasminogen

to plasmin by plasminogen activators causes a conformational change allowing the mature plasmin molecule to degrade fibrin. In addition to fibrin degradation, plasmin also activates matrix metalloproteinases (MMPs) that, in turn, degrade the extracellular matrix [Collen and Lijnen 1991; Birkedal-Hansen 1995].

1.1.1.2 Plasminogen Activators

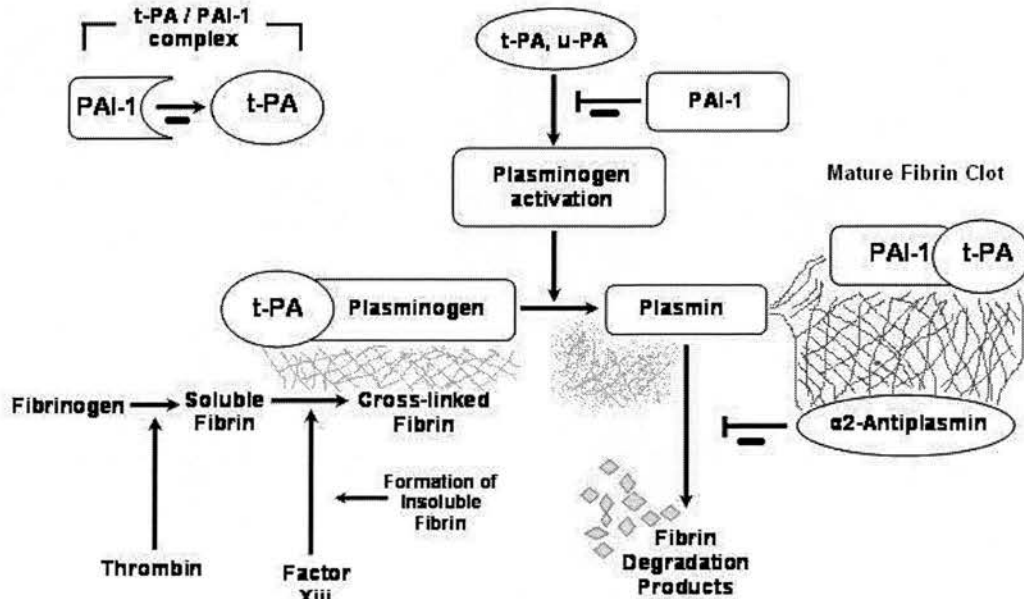
Plasminogen activators cause thrombus dissolution by initiating fibrinolysis. Two physiological plasminogen activators have been identified: (1) t-PA and (2) u-PA which binds to a cellular u-PA receptor (u-PAR). Tissue plasminogen activator is primarily involved in fibrin degradation within the circulatory system whilst u-PA is predominantly involved in cell migration and tissue remodelling [Astedt 1979].

1.1.2 INHIBITION OF FIBRINOLYSIS

The superfamily of serine protease inhibitors (serpins) are a multifunctional group of peptides which play a central role in regulating fibrinolysis as well as coagulation, and inflammation [Potempa *et al* 1994]. All serpins form an irreversible complex with the active site of the target protease with both protease and inhibitor losing their activity. In man, the main plasmin inhibitor is α 2-antiplasmin though other factors may play a role. Systemic unbound plasmin is rapidly inhibited by α 2-antiplasmin, whereas fibrin-bound plasmin is relatively protected from inactivation since the α 2-antiplasmin binding site is occupied by fibrin itself [Wiman *et al* 1979]. There are a number of plasminogen activator inhibitors in man, the most important of which is PAI-1. These inhibitors prevent excessive plasminogen activation by t-PA and u-PA.

Figure 1.1 Activation and inhibition of the endogenous fibrinolytic system; adapted from Kohler and Grant, 2000 [Kohler and Grant 2000].

t-PA - tissue plasminogen activator; PAI-1 - plasminogen activator inhibitor type-1; u-PA - urokinase plasminogen activator; — Inhibition.



1.2 TISSUE PLASMINOGEN ACTIVATOR

Tissue plasminogen activator is the central enzyme responsible for the regulation of endogenous fibrinolysis and removal of intravascular thrombus in humans. After initiation of thrombus formation, the endothelium acutely releases t-PA in response to a range of factors predominantly related to the coagulation cascade, especially factor Xa and thrombin [Emeis 1992].

1.2.1 PROTEIN STRUCTURE AND FUNCTION

Tissue type plasminogen activator consists of 530 amino acids and is composed of several domains with homologies to other proteins including a finger domain, an epidermal growth factor domain, two kringle domains and the protease domain comprising the catalytic triad causing plasminogen activation. Binding of t-PA to fibrin is most likely mediated via the finger and the second kringle domains [Collen 1999]. The human t-PA gene is localised to chromosome 8 with the proximal promoter sequences containing typical TATA and CAAT boxes as well as recognition sequences for transcription factors e.g. Sp1 [Leonardsson and Ny 1997].

1.2.2 TISSUE PLASMINOGEN ACTIVITY

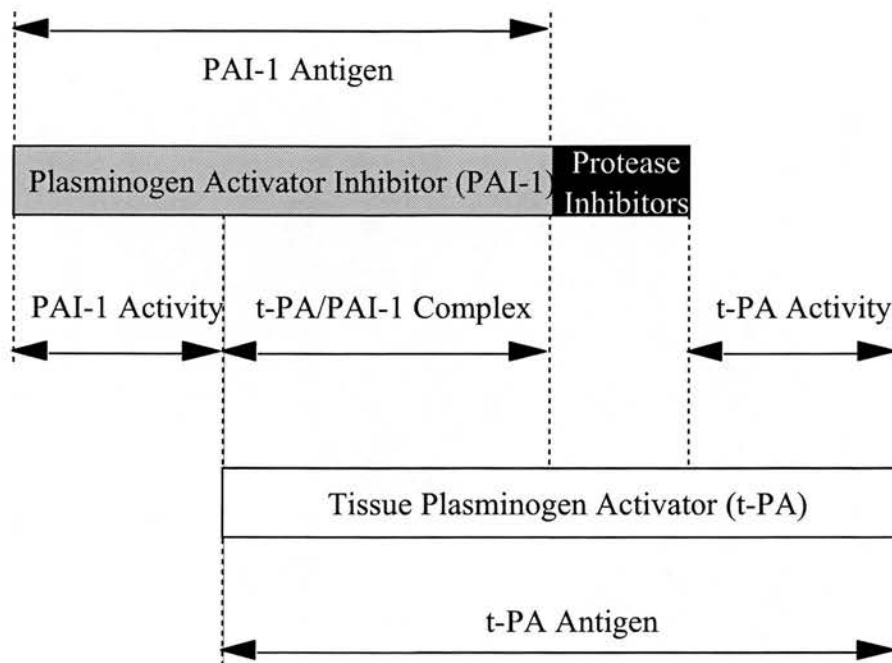
In the absence of fibrin, t-PA has very weak activity as a plasminogen activator. However the conversion of plasminogen to plasmin by t-PA is accelerated 1,000-fold due to conformational changes in the t-PA molecule, in the presence of fibrin and at the endothelial cell surface [Hoylaerts *et al* 1982; Ranby 1982; van Zonneveld *et al* 1986]. Fibrin degradation also results in the appearance of new binding sites for plasminogen on fibrin ensuring efficient localised activation. The efficacy of plasminogen activation and fibrin degradation is determined by the relative balance between the acute local release of t-PA and its subsequent inhibition by PAI-1. Because plasminogen is present at vast molar excess over t-PA in plasma, the onset and efficacy of fibrinolysis are principally determined by the rapidity and magnitude of t-PA release [Hrafnkelsdottir *et al* 2004a].

1.2.3 CIRCULATING t-PA AND t-PA/PAI-1 COMPLEXES

The concentration of t-PA in human plasma is around 3 to 10 ng/mL, though in the resting state only a relatively small proportion is functionally active principally due to the rapid formation of complexes with PAI-1 (Figure 1.2) [Meade *et al* 1993; Nordenhem and Wiman 1998]. There is a pronounced circadian variation in fibrinolytic activity with a peak in t-PA antigen concentrations early in the morning corresponding to low t-PA activity as a consequence of the corresponding 4-fold rise in PAI-1 concentrations [Andreotti and Kluft 1991]. The proportion of active t-PA thus varies inversely with plasma PAI-1 concentration, ranging from 2% to 33% [Chandler *et al* 1990].

The clearance of t-PA occurs through the removal of active t-PA and t-PA/PAI-1 complexes from the circulation by the liver [Chandler *et al* 1997]. This clearance process is rapid, with plasma half-lives for active t-PA and t-PA/PAI-1 complexes of 2.4 and 5.0 minutes respectively. In the presence of high PAI-1 activity, total clearance is slowed due to the longer half-life of the t-PA/PAI-1 complex.

Figure 1.2 Relationship between t-PA and PAI-1 antigen and activity [Oliver *et al* 2005].
t-PA – tissue plasminogen activator; PAI-1 – plasminogen activator inhibitor type-1.



1.2.4 TISSUE PLASMINOGEN ACTIVATOR SYNTHESIS AND RELEASE

The endothelium is the principal site of t-PA generation where it is released via both constitutive and regulated pathways [van den Eijnden-Schrauwen *et al* 1995]. Although present within most endothelial cells, the synthesis and secretion of t-PA is greatest within those lining precapillary arterioles and postcapillary venules and is minimal within the major vessels of the aorta [Levin and del Zoppo 1994]. There are regional differences in endothelial t-PA release such that the upper limbs release four times the amount of the lower limbs [Keber 1988]. Circulating haematopoietic cells contribute to the production of t-PA within atherosclerotic plaques and may be important in the degradation of extracellular matrix and plaque instability [Lupu *et al* 1995; Steins *et al* 1999].

The mechanisms regulating synthesis of t-PA are not fully understood though synthesis is increased by thrombin, prostacyclin and histamine [Hanss and Collen 1987] and reduced by plasmin [Shi *et al* 1992]. Acute stimulated release of t-PA occurs with rapid translocation of a dynamic intracellular storage pool in response to blood coagulation and humoral factors [Emeis 1992; van den Eijnden-Schrauwen *et al* 1995]. The acute capacity of the endothelium to release t-PA is substantial with continuous release demonstrated over several hours without significant tachyphylaxis [Wall *et al* 1997; Newby *et al* 1998] yielding forearm t-PA concentrations approaching those following administration of thrombolysis in acute myocardial infarction (MI) [Oliver *et al* 2005].

1.2.5 MOLECULAR CONTROL OF T-PA RELEASE

In cell culture, constitutive release of t-PA from the endothelium is augmented by protein kinase C (PKC) activation and in PKC-activated cells, cyclic adenosine monophosphate (cAMP) agonists augment t-PA secretion [Santell and Levin 1988]. Thrombin and prostacyclin induce a rapid and dose-dependent release of t-PA through independent G-protein pathways involving G_q /intracellular calcium concentrations and G_s /cAMP dependent pathways respectively [Tranquille and Emeis 1991a; van den Eijnden-Schrauwen *et al* 1997; Hegeman *et al* 1998; Knop and Gerke 2002]. In healthy volunteers, basal t-PA release appears to be modified by genetic variation at the t-PA locus [Jern *et al* 1999; Ladenvall *et al* 2001]. Tissue plasminogen activator genotype might therefore contribute to inter-individual differences in the endogenous fibrinolytic capacity and the risk of arterial thrombotic events.

Endothelial-dependent agonists such as substance P [Newby *et al* 1997], bradykinin [Brown *et al* 1999; Labinjoh *et al* 2000] and methacholine [Jern *et al* 1994; Wall *et al* 1997] cause a rapid release of t-PA *in vivo*. This is independent of changes in blood flow *per se* since infusion of sodium nitroprusside to increase forearm blood flow (FBF) by more than 600% does not affect t-PA release [Newby *et al* 1997; Stein *et al* 1998]. Although shear stress increases t-PA expression and the intracellular storage pool, it does not cause appreciable t-PA release *in vivo* [Sjogren *et al* 2000]. In addition, norepinephrine releases t-PA while reducing blood flow [Jern *et al* 1994] and intra-arterial infusion of tumour necrosis factor- α (TNF- α), induces t-PA release without changing blood flow [Chia *et al* 2003b]. Thus blood flow and shear stress appear to regulate endothelial t-PA synthesis and storage but do not affect its acute release.

1.3 PLASMINOGEN ACTIVATOR INHIBITOR TYPE-1

1.3.1 PROTEIN STRUCTURE AND FUNCTION

Plasminogen activator inhibitor type-1 is a glycoprotein containing 379 amino acids and has a plasma concentration of ~500 pmol/L. There is a several-fold molar excess of PAI-1 over t-PA in plasma [Chandler *et al* 1990] to which it rapidly binds forming a stable complex with a ratio of 1:1. Plasminogen activator inhibitor type-1 promotes the stability and extension of thrombus by preventing t-PA binding to fibrin. The active form of PAI-1 is unstable with a half-life of about 30 minutes [Kooistra *et al* 1986]. Inhibition of t-PA by PAI-1 is reduced ~90% in the presence of fibrin due to reduced access to the catalytic domain of fibrin-bound t-PA [Keijer *et al* 1991]. As

the complex structure of polymerised fibrin decreases access of t-PA to fibrin, the efficacy of fibrinolysis is greatest in the early phase of fibrin formation on the surface of atheromatous plaques. In contrast, fully formed and cross-linked fibrin is resistant to t-PA mediated fibrinolysis [Thorsen 1992].

1.3.2 SYNTHESIS AND RELEASE

The majority of PAI-1 synthesis and release occurs in vascular tissue, namely endothelium and vascular smooth muscle, although the liver and platelets provide further contributions [Sprengers and Kluft 1987]. Thrombin stimulates PAI-1 synthesis within endothelial cells and expression on the surface of activated platelets. Platelet-rich thrombi are more susceptible to lysis by t-PA in PAI-1 deficient mice suggesting platelets may provide a significant local source of PAI-1 [Higgins and Vehar 1987].

1.3.3 PAI-1 POLYMORPHISMS

The human PAI-1 gene located on chromosome 7 contains nine exons and eight introns [Strandberg *et al* 1988]. Several polymorphisms have been described including a common four or five guanine base pair polymorphism in the promoter region upstream of the transcription start site (4G/5G). Subjects homozygous for the 4G allele have increased gene transcription and increased plasma levels of PAI-1 [Eriksson *et al* 1995]. There appears to be association of the 4G/4G genotype with an increased risk of coronary disease [Iacoviello *et al* 1998] though this may reflect interaction between PAI-1 4G/5G genotype, plasma hypertriglyceridaemia and the insulin resistance syndrome [Kohler and Grant 2000].

1.4 ENDOTHELIAL FUNCTION

The endothelium plays a vital role in the regulation of blood flow, coagulation and fibrinolysis as well as the response to tissue injury and inflammation. This vascular homeostasis is maintained through the synthesis and release of a number of factors from the endothelium in response to mechanical, neural and humoral stimuli (Table 1.1).

1.4.1 REGULATION OF VASOMOTION

The pioneering work of Furchgott and Zawadzki [Furchgott and Zawadzki 1980] led to the subsequent description of nitric oxide (NO) as the prototypical endothelial-derived vasodilator. It is now recognised that a number of such factors are physiologically active within the human circulation (Table 1.1). A number of endogenous factors including bradykinin and substance P rely on the integrity of the endothelium to mediate vascular smooth muscle relaxation. An important component of such endothelium-dependent smooth muscle relaxation consists of calcium-dependent activation of the constitutive enzyme NO synthase (NOS) and release of NO [Moncada and Higgs 1993]. The relative dependency on NO, endothelium-derived hyperpolarising factor (EDHF) and prostacyclin-mediated vasodilatation depends on the specific stimulus as well as the basal resting conditions.

As well as regulating vasodilatation, the endothelium also synthesises and responds to vasoconstricting factors including endothelin-1 [Webb 1995a] and angiotensin II

[Kifor and Dzau 1987]. The continual basal release of NO and endothelin plays an important role in maintaining vascular tone and blood pressure [Vallance *et al* 1989; Haynes *et al* 1996].

Table 1.1 Factors secreted and regulated by the endothelium to maintain vascular haemostasis

Regulation of vascular tone	
Nitric oxide	Endothelin-1
Prostaglandins, thromboxane A2	Angiotensin II
Endothelium derived hyperpolarising factor (EDHF)	
Haemostasis and fibrinolysis	
Tissue plasminogen activator	Plasminogen activator inhibitor-1
Heparins	Tissue factor
Thrombomodulin	Von Willebrand factor
Inflammatory factors	
Monocyte chemotactic factor-1 (MCP-1)	
Adhesion molecule expression (VCAM-1, ICAM-1, selectins)	
Interleukins 1, 6, and 18	
Tumour necrosis factor- α	

1.4.2 COAGULATION AND ENDOGENOUS FIBRINOLYSIS

The endothelium is the source of a number of coagulation and fibrinolytic factors. Although the intact endothelial monolayer maintains an antithrombotic surface, arterial injury is associated with the development of a pro-coagulant phenotype. The regulation of the endogenous fibrinolytic system has been reviewed above and its role in the pathophysiology of coronary heart disease (CHD) will be discussed further.

1.5 ENDOTHELIAL FUNCTION AND ATHEROTHROMBOSIS

Atherosclerosis is associated with a decrease in the synthesis and release of endothelially-derived factors with an increased risk of local thrombus formation and reduced blood flow risking vessel occlusion and tissue infarction. Nearly all of the candidate risk factors for atherosclerosis appear detrimental to endothelial function.

1.5.1 ENDOTHELIUM DEPENDENT VASODILATOR RESPONSES

To date, most clinical studies have focused on the assessment of endothelium-dependent vasomotion as a surrogate measure of endothelial function. This can be undertaken by measuring resistance vessel responses to endothelial stimulants as well as conduit vessel responses to reactive hyperaemia or pharmacological stimulation. Invasive and non-invasive methods of assessing endothelial function have been shown to be highly reproducible with low coefficients of variation reported for both strain gauge plethysmography and flow-mediated dilatation of the brachial artery [Tousoulis *et al* 2005].

The endothelium-dependent regulation of peripheral vascular tone is impaired by atherosclerosis and its risk factors such as smoking [Celermajer *et al* 1996; Heitzer *et al* 1996; Newby *et al* 1999; Pretorius *et al* 2002], hypercholesterolaemia [Celermajer *et al* 1992; Stroes *et al* 1995], diabetes mellitus [Calver *et al* 1992] and a familial history of atherosclerosis [Clarkson *et al* 1997]. Similarly, impaired vasodilator responses within the coronary circulation in patients with coronary disease [Ludmer *et al* 1986] and its risk factors including smoking [Zeicher *et al* 1995] suggest

impaired endothelial responses are a systemic feature of atherosclerosis. Furthermore, impaired endothelium-dependent vasomotor dilatation in the peripheral [Heitzer *et al* 2001; Perticone *et al* 2001] and coronary [Suwaidi *et al* 2000; Halcox *et al* 2002b] circulations, independently predicts the risk of future adverse cardiovascular events including subjects without angiographical evidence of significant coronary disease [Suwaidi *et al* 2000] (Table 1.2). Whilst providing an important indicator of endothelial function, the pathophysiological mechanism linking impaired endothelium-dependent smooth muscle relaxation and subsequent atherothrombotic events remains unclear.

Table 1.2 Predictive value of impaired endothelium-dependent vasodilatation and risk of cardiovascular events adapted from Widlansky et al 2003 [Widlansky *et al* 2003]

Patient Population	Study Design	Vascular Bed	Marker of function	End Points	Findings
157 pts with mild CAD [Suwaidi <i>et al</i> 2000]	Retrospective/ 28 months	Coronary	Ach response	CV Death, MI, CCF or PCI	Ach responses independent predictor of events
147 pts with CAD [Schachinger <i>et al</i> 2000]	Retrospective/ 7.7 years	Coronary	Ach response, FMD	MI, UA, CVA, CABG, PCI, PVD	Vasomotor response independent predictor of events
281 pts with CAD [Heitzer <i>et al</i> 2001]	Prospective/ 4.5 years	Brachial	Ach response	CV Death, CVA, MI, CABG, PCI, PVD	Ach responses independent predictor of events
225 pts with hypertension [Perticone <i>et al</i> 2001]	Prospective/ 32 months	Brachial	Ach response	CV Death, CVA, TIA, MI, UA, CABG, PCI, PVD	Ach responses independent predictor of events
308 pts undergoing angiography [Halcox <i>et al</i> 2002b]	Retrospective/ 46 months	Coronary	Ach response	CV Death, CVA, MI, UA	Ach responses independent predictor of events

Ach - acetylcholine; CABG - coronary artery bypass graft surgery; CAD - coronary artery disease; CCF - congestive cardiac failure; CVA - ischaemic stroke; CV death - death from a cardiovascular death; FMD - flow-mediated dilation; MI - myocardial infarction; PCI - percutaneous coronary intervention (angioplasty or stent); PVD - peripheral vascular disease; TIA - transient ischaemic attack; UA - unstable angina.

1.5.2 IN VIVO ASSESSMENT OF ACUTE T-PA RELEASE

In humans, acute release of t-PA can be assessed systemically following venous occlusion [Gris *et al* 1991], intravenous infusion of bradykinin [Brown *et al* 1997] or in response to exercise [Chandler *et al* 1992]. However, these approaches are limited by potential confounding effects, such as changes in systemic haemodynamics, activation of the sympathetic nervous system, clearance of t-PA and PAI-1, and concomitant release of other mediators. Direct assessment of local capacity for acute t-PA release within individual vascular beds avoids these problems and is likely to better represent the defence against arterial thrombosis [Oliver *et al* 2005].

A near instantaneous release of endothelial t-PA is seen during intra-arterial substance P [Newby *et al* 1997; Newby *et al* 1999], bradykinin, [Brown *et al* 1999; Witherow *et al* 2002] and methacholine [Stein *et al* 1998] infusions with impaired release of t-PA seen in cigarette smokers [Newby *et al* 1999; Pretorius *et al* 2002] and patients with CHD [Newby *et al* 2001]. Although the forearm circulation is relatively protected from the development of atheroma, the acute fibrinolytic responses in the forearm [Newby *et al* 1999; Labinjoh *et al* 2001a; Witherow *et al* 2002] and coronary [Minai *et al* 2001; Newby *et al* 2001; Matsumoto *et al* 2003] circulations appear similar in subjects with cardiac disease.

1.5.3 VASCULAR INFLAMMATION

Atherosclerosis is a complex inflammatory process involving numerous cell types [Ross 1999]. Elevated levels of inflammatory markers such as C-reactive protein (CRP) [Liuzzo *et al* 1994; Haverkate *et al* 1997; Ridker *et al*

1997b] and interleukin-6 [Ridker *et al* 2000b] predict the occurrence of adverse cardiovascular events independent of other conventional risk factors. Acute inflammation has recently been shown to directly affect endothelial vasomotor [Hingorani *et al* 2000b] and fibrinolytic function [Chia *et al* 2003b] in healthy volunteers suggesting that inflammatory mediators contribute to vascular dysfunction. In keeping with this hypothesis, anti-inflammatory therapies may ameliorate inflammation-induced endothelial dysfunction [Kharbanda *et al* 2002].

1.6 ENDOGENOUS FIBRINOLYSIS AND CORONARY HEART DISEASE

1.6.1 PLASMA LEVELS OF FIBRINOLYTIC FACTORS AND RISK OF CORONARY DISEASE

In epidemiological studies of patients with ischaemic heart disease [Thompson *et al* 1995; Juhan-Vague *et al* 1996; Wiman *et al* 2000], and in prospective studies in healthy populations [Ridker *et al* 1993; van der Bom *et al* 1997; Lowe *et al* 1998; Thogersen *et al* 1998], higher total plasma t-PA concentrations positively and independently predict future coronary events. However it would be anticipated that high t-PA concentrations would protect against subsequent cardiovascular events rather than the reverse.

There are several plausible mechanisms that may explain the observed relationship between increased t-PA levels and risk of cardiovascular events. Increased t-PA antigen levels are associated with increased levels of PAI-1 antigen and circulating

t-PA/PAI-1 complexes [Carter *et al* 1998; Wiman *et al* 2000] thereby reducing overall free t-PA 'activity' [Jansson *et al* 1993]. Secondly, areas of endothelial denudation and thrombus deposition are a common finding on the surface of atheromatous plaques and are usually subclinical [Davies 2000a]. The adverse prognosis conferred by elevated plasma t-PA antigen concentrations may, therefore, reflect the extent of occult atheroma and subclinical plaque rupture stimulating t-PA release. Although effective t-PA release may prevent thrombus propagation, organisation of the residual thrombus may lead to plaque growth and expansion increasing the overall burden of atheroma [Mann and Davies 1999]. The increase in systemic circulating t-PA may be linked to enhanced t-PA gene expression observed within atherosclerotic vessels including aortic aneurysms and atheromatous plaques [Lupu *et al* 1995; Schneiderman *et al* 1995]. Plasminogen activator mRNA expression is greatest in advanced macrophage and lipid-rich plaques and in areas of lesion necrosis [Lupu *et al* 1995; Steins *et al* 1999] suggesting that higher t-PA levels might be associated with a greater risk of unstable plaque rupture secondary to excess fibrinolytic activity.

1.6.2 ACUTE TISSUE PLASMINOGEN ACTIVATOR RELEASE AND RESOLUTION OF THROMBUS

Although plaque related microthrombi are a common finding, effective local t-PA release may result in these remaining subclinical and becoming quiescent. However deficiencies in t-PA release may cause such microthrombi to propagate, ultimately leading to arterial occlusion. Once thrombotic occlusion has occurred, the probability of reperfusion occurring will be determined by the efficacy of endogenous

fibrinolysis. Indeed, following acute MI and in the absence of reperfusion therapy, the infarct-related artery undergoes spontaneous reperfusion in around 30% of patients within the first 12 hours [DeWood *et al* 1980; Armstrong *et al* 1989; Rentrop *et al* 1989]. If endogenous fibrinolysis is to be effective, then the rapid mobilisation of t-PA from the endothelium is essential because thrombus dissolution is much more effective if t-PA is incorporated during, rather than after, thrombus formation [Fox *et al* 1985]. As the rapidity and extent of acute t-PA release from the endothelium is the principal determinant of the efficacy of local endogenous fibrinolysis [Hrafnkelsdottir *et al* 2004a; Oliver *et al* 2005], the capacity of the endothelium to release t-PA might predict the clinical outcome of individual plaque events and long-term cardiovascular risk.

1.6.3 ACUTE T-PA RELEASE, REGULATION OF VASCULAR TONE AND RISK OF CARDIOVASCULAR EVENTS

Previous work has demonstrated impaired forearm t-PA release in cigarette smokers [Newby *et al* 1999], a group who are at particular risk of coronary thrombosis and MI [Burke *et al* 1997]. This suggests that endogenous fibrinolysis plays an important role in the pathogenesis of coronary thrombosis. Reports of preserved endothelium-dependent vasodilatation in smokers [Pretorius *et al* 2002] and in patients with hypertension [Hrafnkelsdottir *et al* 1998; Hrafnkelsdottir *et al* 2004b] despite reduced acute t-PA release suggest that, in some circumstances, reduced t-PA release may be a more sensitive marker of endothelial dysfunction. However at present, the relationship between the capacity to release t-PA and the future risk of adverse cardiovascular events is unknown.

1.7 GENETIC POLYMORPHISMS AND ATHEROSCLEROSIS

The distribution of atherothrombotic disease amongst individuals and populations results from the interaction between susceptibility genes and environmental exposures which combine to produce an overall disease phenotype.

1.7.1 GENETIC FACTORS AND CARDIOVASCULAR RISK

After correction for traditional risk factors such as hypertension, hyperlipidaemia and smoking, there remains a significant genetic influence on the risk of developing symptomatic coronary disease [Marenberg *et al* 1994; de Lange *et al* 2001]. Indeed, first-degree relatives of patients with premature coronary disease are themselves at an increased risk of developing coronary disease at an early age [Shea *et al* 1984; Schildkraut *et al* 1989; Steeds and Channer 1997]. The familial risk of CHD could be due to shared genetic or environmental factors, or more likely a combination of both. Interestingly, Scandinavian twin registry studies reported an increased concordance for risk of death from CHD in monozygotic twins compared with dizygotic twins, suggesting that sharing genes is more important than sharing family environment in determining mortality from cardiovascular disease [Marenberg *et al* 1994].

1.7.2 SINGLE NUCLEOTIDE POLYMORPHISMS

Although single mendelian gene defects predisposing to early onset coronary disease such as familial hypercholesterolaemia do exist, more commonly cardiovascular disease manifests as a late onset condition without a clear mode of inheritance [Hingorani 2000a]. There is increasing interest in the paradigm that common genetic variants with a >1% carrier frequency in the general population might explain the predisposition to common conditions such as CHD, stroke and hypertension. These single nucleotide polymorphisms (SNPs) are single base pair positions within genomic DNA and comprise about 90% of human DNA sequence variation [Li and Sadler 1991] occurring at a frequency of about 1 per 1,000 base pairs [Collins *et al* 1998; Brookes 1999].

1.7.3 GENETIC POLYMORPHISMS OF THE HAEMOSTATIC SYSTEM

As the final common pathway of many cardiovascular diseases involves thrombus formation there is great interest in how genetic regulation of haemostasis may contribute to the pathogenesis of vascular events by altering the production or metabolism of haemostatic factors. Elevated fibrin D-dimer and t-PA antigen concentrations have been reported in male, healthy relatives of subjects with severe CHD [Mills *et al* 2002], with twin studies suggesting genetic factors account for approximately 60% of the inter-individual variation in t-PA and PAI-1 antigen levels [de Lange *et al* 2001]. A number of haemostatic factor and platelet glycoprotein receptors linked to thrombotic risk are shown (Table 1.3).

Table 1.3 Polymorphisms in the haemostatic system linked to the risk of arterial thrombosis adapted from Voetsch and Loscalzo 2004 [Voetsch and Loscalzo 2004]

Fibrinolysis	Coagulation	Platelet Receptor
t-PA <i>Alu</i> -Ins/Del	Fibrinogen β -455 G/A	GPIIIa Leu33Pro
t-PA -7,351 C/T	Fibrinogen β -854 G/A	GP1b α VNTR
PAI-1 4G/5G	Fibrinogen α Thr312Ala	GP1b α Thr145Met
TAFI Ala147Thr	Factor V Leiden	GP1a/IIa α 2 807C/T

t-PA - tissue plasminogen activator; Ins - insertion; Del - deletion; PAI-1 - plasminogen activator inhibitor type-1; TAFI - thrombin activatable fibrinolysis inhibitor.

1.7.4 TISSUE PLASMINOGEN ACTIVATOR GENETIC POLYMORPHISMS

A common intronic polymorphism of the t-PA gene is generated by the insertion of a 311 base pair *Alu*-repeat sequence [Ludwig *et al* 1992] with a similar polymorphism in the angiotensin-converting enzyme (ACE) gene reportedly explaining 50% of the variability of plasma ACE levels between individuals [Rigat *et al* 1990]. Although not a consistent finding in all populations [Ridker *et al* 1997a], there is an independent association between the insertion (Ins) genotype and the future risk of MI: a relative risk of 2.24 in comparison with the deletion (Del) genotype [van der Bom *et al* 1997].

In young healthy volunteers, basal t-PA release from the forearm circulation correlates with t-PA *Alu* genotype with Ins homozygous subjects having greater basal release than either heterozygotes or Del homozygotes [Jern *et al* 1999]. The nature of the Ins/Del polymorphism, an insertion of an *Alu*-repeat in an intron, makes a direct functional effect of the insertion allele on the t-PA protein unlikely but not impossible [Makalowski *et al* 1994]. Three further polymorphisms at the t-PA locus

are in significant linkage disequilibrium with the *Alu* polymorphism and consequently associated with t-PA release rates: one in the far upstream enhancer (7,351 C→T substitution), one in exon 6 (20,099 T→C), and one in intron 10 (27,445 T→A) [Ladenvall *et al* 2000]. The -7,351 C/T enhancer SNP resides within a GC box which is a binding site for the transcription factor Sp1 with reduced binding affinity of Sp1 in the presence of the T allele [Ladenvall *et al* 2000]. As the T allele is associated with low t-PA release rate, this suggests the -7,351 C/T variant is important in regulating t-PA levels at the level of transcription [Ladenvall *et al* 2000; Wolf *et al* 2005]. Recently, -7,351 TT homozygotes have been shown to be at increased risk of both MI [Ladenvall *et al* 2002] and lacunar stroke [Jannes *et al* 2004] compared to CC homozygotes.

Inter-individual differences in t-PA release due to genetic variation at the t-PA locus may modify the fibrinolytic response to local thrombus formation. Although the effect of t-PA polymorphisms has been assessed in asymptomatic individuals, the functional importance of t-PA genotype on acute fibrinolytic capacity in patients with established CHD and its associated risk factors is unknown.

1.8 ACUTE INFLAMMATION AND ENDOTHELIAL FUNCTION IN PATIENTS WITH CORONARY DISEASE

1.8.1 INFLAMMATION AND ATHEROSCLEROSIS

Plasma markers of systemic inflammation, such as CRP and TNF- α , are elevated in patients with cardiovascular disease [Haverkate *et al* 1997; Ridker *et al* 2000a] and

predict the development of cardiovascular disease independent of other risk factors. Increased levels of CRP in those at greatest risk may reflect a larger total burden of atheroma, a greater inflammatory activity predisposing to plaque rupture, or a combination of these mechanisms [Davies 2000a].

Focal arterial inflammation is one of the most prominent characteristics of the atherosclerotic process [Ross 1999] including the pathogenesis of acute coronary syndromes [Davies 2000a] with systemic release of thromboxanes and leukotrienes, and activation of circulating leucocytes [Neumann *et al* 1997; Aukrust *et al* 1999]. Indeed, a process of infiltration by mononuclear leucocytes followed by fibrosis and finally tissue degeneration is common to many other inflammatory disorders apart from atherosclerosis [Tousoulis *et al* 2003]. In keeping with this observation, subjects with the auto-immune inflammatory disorder rheumatoid arthritis are at an increased risk of cardiovascular morbidity and mortality [Mutru *et al* 1985].

Human recombinant CRP, at concentrations known to predict vascular disease, contributes to the development of a pro-inflammatory and pro-atherosclerotic endothelial phenotype characterised by expression of cell adhesion molecules [Pasceri *et al* 2000; Pasceri *et al* 2001], downregulation of endothelial NO synthase (eNOS) expression [Verma *et al* 2004] and production of interleukin-6 [Verma *et al* 2000]. C-reactive protein has recently been shown to protect endothelial cells from complement mediated injury suggesting the development of atherosclerosis may depend on a balance of its pro- and anti-atherogenic actions [Verma *et al* 2004].

1.8.2 INFLAMMATION AND HAEMOSTATIC FACTORS

Although elevated levels of t-PA independently predict the occurrence of cardiovascular events [Ridker *et al* 1993; Thompson *et al* 1995; van der Bom *et al* 1997; Lowe *et al* 1998; Thogersen *et al* 1998], t-PA concentrations may be implicated in the mechanisms contributing to, or arise as a consequence of, atherothrombotic events. Previous studies have indicated a direct relationship between serum CRP and plasma t-PA concentrations [Haverkate *et al* 1995; Juhan-Vague *et al* 1996; Gram *et al* 2000; Lowe *et al* 2004]. This raises the question of whether vascular inflammation is causally related to the elevation in plasma t-PA concentrations or whether CRP and t-PA are independently increased by a common factor related to the atherosclerotic process itself, such as acute plaque rupture.

1.8.3 VASCULAR INFLAMMATION AND ENDOTHELIAL FUNCTION

In healthy volunteers, experimental inflammation has been shown to impair endothelium-dependent dilatation in the arterial [Hingorani *et al* 2000b; Chia *et al* 2003b] and venous [Bhagat and Vallance 1999] circulations. Pre-treatment with an anti-inflammatory dose of aspirin inhibits the inflammation-induced rise in interleukin-1 receptor antagonist levels and impaired endothelium-dependent vasodilator responses [Kharbanda *et al* 2002]. Together, these observations suggest that pro-inflammatory cytokines directly affect endothelial function.

In patients with coronary artery disease, elevated serum concentrations of CRP are associated with impaired forearm endothelial vascular reactivity [Fichtlscherer *et al* 2000]. Furthermore, normalisation of CRP levels over time is associated with a

significant improvement in endothelium-dependent FBF responses [Fichtlscherer *et al* 2000] whereas ongoing chronic inflammation is associated with an impaired fibrinolytic response to venous occlusion [Speidl *et al* 2005]. Recently, intra-arterial infusion of the pro-inflammatory cytokine in healthy volunteers was shown to impair endothelium-dependent vasodilatation whilst increasing local t-PA release [Chia *et al* 2003b]. This suggests that vascular inflammation has discrete and regulated effects on endothelial function causing a simultaneous reduction in blood flow whilst potentiating endogenous fibrinolysis. As the endothelium is the major source of plasma t-PA, abnormalities of endothelial function may therefore mediate the potential inflammation-induced elevations in plasma t-PA concentrations and underlie the link between t-PA levels and future vascular events. However, the effect of acute arterial inflammation in subjects with pre-existing vascular dysfunction and coronary disease is unknown.

1.9 NITRIC OXIDE AND ENDOTHELIAL DYSFUNCTION

The normal endothelium, by releasing NO, promotes vasodilation and inhibits inflammation, thrombosis, and vascular smooth muscle cell proliferation. Although originally described as a vasodilator [Furchgott and Zawadzki 1980; Ignarro 1989], these biological actions of NO make it an important component in the defence against atherosclerosis.

1.9.1 ENDOTHELIAL NITRIC OXIDE SYNTHESIS

A number of vasodilators have since been shown to depend on the integrity of the vascular endothelium and NO release for their activity, including substance P [Newby *et al* 1998] and bradykinin [Cockcroft *et al* 1994b]. An important component of such endothelium-dependent responses consists of calcium-dependent activation of the constitutive enzyme eNOS, which catalyses the conversion of the amino acid L-arginine to L-citrulline and NO [Moncada and Higgs 1993]. Constitutive NOS can be competitively inhibited by guanidine-substituted analogues of L-arginine, such as L-N^G-monomethyl arginine (L-NMMA). Inorganic nitrates, such as sodium nitroprusside, can activate the same effector pathway by providing an inorganic source of NO which is not dependent on the functional integrity of the vascular endothelium.

1.9.2 NITRIC OXIDE AVAILABILITY AND ATHEROSCLEROSIS

Reduced nitric oxide availability may be due to (1) decreased endothelial NO production, (2) increased levels of endogenous NO inhibitors or (3) increased destruction of NO by free radicals [Vallance 2003]. Deficiency of biologically active NO favours leucocyte adhesion to the injured endothelium, platelet aggregation and smooth muscle migration and proliferation increasing atheroma formation [Cayatte *et al* 1994] and the risk of thrombus propagation [Ganz and Vita 2003]. In animal models, gene transfer of eNOS is associated with a reduction in neo-intimal formation following arterial injury [Varenne *et al* 2000] whilst in humans supplementation with L-arginine, the endogenous substrate for NO, restores

endothelium-dependent vasodilator responses in hypercholesterolaemic patients [Drexler *et al* 1991].

1.9.3 NITRIC OXIDE AND GUANYLATE CYCLASE ACTIVATION

Once synthesised, NO diffuses to the underlying vascular smooth muscle where it activates soluble guanylate cyclase increasing cyclic guanosine monophosphate (cGMP) concentrations [Ignarro 1989; Moncada and Higgs 1993]. The physiological cell signalling actions of NO are predominantly due to increased cGMP which occurs with NO concentrations in the nanomolar range [Vallance 2003]. At higher levels NO has the potential to nitrosylate cysteine residues in proteins [Stamler *et al* 2001] as well as affecting a variety of haem-containing and redox sensitive enzymes and ion channels [Vallance 2003].

1.9.4 PHOSPHODIESTERASE ENZYMES

Phosphodiesterases (PDEs) were identified as being responsible for the hydrolysis of cyclic nucleotides over 40 years ago [Sutherland 1972]. Phosphodiesterases hydrolyse cGMP to guanosine monophosphate (GMP) and several isoenzymes exist, each with a different tissue distribution and specificity for cGMP [Wallis *et al* 1999; Mehats *et al* 2002].

1.9.4.1 Phosphodiesterase Type 5 Inhibition

Phosphodiesterases negatively regulate the action of NO by degrading cGMP to GMP. Phosphodiesterase isoenzyme type 5 (PDE 5) is the enzyme responsible for inactivation of cGMP and is found in high concentrations in the corpus cavernosum,

skeletal muscle, platelets and vascular smooth muscle [Mehats *et al* 2002]. One approach to ameliorate some of the deleterious effects of reduced NO release and bioavailability is to prolong the actions of cGMP through inhibition of PDE 5. Sildenafil citrate (Viagra[®] Pfizer Ltd) is a selective PDE 5 inhibitor used in the treatment of erectile dysfunction. Erectile dysfunction is particularly common in patients with heart disease, because of the presence of overlapping risk factors, including older age, diabetes mellitus, hypertension, and hypercholesterolaemia [Feldman *et al* 1994]. As sildenafil therapy increases NO levels within the corpus cavernosum, it might also have favourable effects on NO balance in the presence of atherosclerotic disease.

1.9.4.2 Phosphodiesterase Inhibition and Endogenous Fibrinolysis

Although substance P-induced t-PA release was inhibited by the NOS inhibitor, L-NMMA [Newby *et al* 1998] sodium nitroprusside does not stimulate t-PA release [Jern *et al* 1994; Newby *et al* 1997; Hrafnkelsdottir *et al* 1998; Brown *et al* 1999]. Thus, although NO alone does not induce t-PA release, it may play a permissive or synergistic role in stimulated t-PA release. As NO mediates many of its actions through cGMP, there is a potential interaction between cGMP and t-PA release. In animal models, non-selective PDE inhibitors such as pentoxifylline and its analogues, increase stimulated but not basal plasma t-PA levels [Tranquille and Emeis 1991b]. Pentoxifylline has also been shown to potentiate the effect of exogenous thrombolysis in the dissolution of fibrin clots [Ambrus *et al* 1994].

There is significant cross-talk between different PDE isoenzymes: phosphodiesterase isoenzyme type 3 (PDE 3), which normally hydrolyses cAMP and is present within vascular smooth muscle, is inhibited by increased cGMP concentrations [Maurice *et al* 2003]. Increased cAMP concentrations levels play a role in thrombin, prostacyclin and isoproterenol-induced acute t-PA release [Hegeman *et al* 1998]. Sildenafil might therefore augment acute t-PA release from endothelial stores via PDE 5-mediated inhibition of PDE 3 and increased intracellular cAMP concentrations.

1.9.4.3 Phosphodiesterase Type 5 Inhibition and Vascular Tone

Intravenous or oral administration of sildenafil results in a small but consistent decrease in systemic and pulmonary blood pressure in both normal men [Jackson *et al* 1999] and those with stable CHD [Jackson *et al* 1999; Herrmann *et al* 2000; Webb *et al* 2000]. This presumably reflects augmentation of the vascular effects of basal NO release mediated through an increase in cGMP. In men with severe coronary artery disease, sildenafil does not alter coronary blood flow [Herrmann *et al* 2000] but augmentation of the coronary hyperaemic response to adenosine in the presence of sildenafil suggests functional interaction between the cAMP and cGMP pathways in humans [Herrmann *et al* 2000].

Oral administration of sildenafil increases vasodilatation during infusion of acetylcholine in the peripheral circulation of healthy cigarette smokers [Kimura *et al* 2003] and in the coronary circulation of patients with CHD [Halcox *et al* 2002a]. Although these results suggest PDE 5 inhibition may improve endothelial-dependent responses, these studies were not placebo-controlled and do not account for the

variation in active plasma concentrations seen following a single oral dose of sildenafil citrate [Boolell *et al* 1996]. In a more recent randomised double-blinded placebo-controlled study in young smokers sildenafil therapy did not change flow-mediated dilatation [Dishy *et al* 2004].

Despite these initial studies on one aspect of endothelial function in healthy subjects, the effects of sildenafil on endogenous t-PA release and levels of PAI-1 in subjects with atherosclerosis are unknown. Therapeutic intervention with PDE 5 inhibitors could represent a novel approach to restoring endothelial responses by prolonging the action of NO and confer secondary preventative benefits in patients with CHD.

1.10 HYPOTHESES

Coronary heart disease and its associated risk factors such as cigarette smoking, are associated with a pro-inflammatory state and increased risk of vascular occlusion secondary to thrombus formation. As tissue plasminogen activator is the principal mediator of endogenous fibrinolysis in humans, acute t-PA release may be a principal determinant of the future risk of atherothrombotic events. Differences in t-PA release between individuals may explain the variation in the burden of cardiovascular events with seemingly similar risk factor profiles. Therapeutic strategies to augment the endogenous fibrinolytic response may be associated with favourable outcomes in patients with CHD.

The following hypotheses will be addressed:

1. Reduced acute release of t-PA will be associated with an increased risk of future atherothrombotic events in patients with established CHD.
2. That genetic variation within the t-PA locus may influence the acute fibrinolytic capacity of patients with CHD.
3. Local vascular inflammation will directly affect endothelial control of vascular tone and fibrinolytic function in patients with CHD.
4. Acute PDE type 5 inhibition will modify t-PA release and vasomotor function in patients with stable CHD.

1.11 AIMS

The aims of the thesis were:

In patients with stable CHD

- To determine whether acute stimulated t-PA release predicts the future risk of adverse atherothrombotic events.
- To investigate whether substance P-induced endothelium-dependent vasodilatation predicts the future risk of adverse atherothrombotic events.

In patients with CHD

- To determine the frequency of four polymorphisms of the t-PA gene in a cohort of unrelated Caucasian subjects.
- To investigate whether the (i) *Alu*-repeat Ins/Del, (ii) C→T enhancer region (-7,351 C/T), (iii) T→C exon 6 (20,099 T/C), and (iv) T→A (27,445 T/A) intron 10 polymorphisms of the t-PA gene influence vasomotor and fibrinolytic function.

In patients with CHD

- To establish the relationship between plasma CRP, t-PA and PAI-1 antigen concentrations.
- To determine whether intra-arterial infusion of the pro-inflammatory cytokine TNF- α results in local vascular inflammation.
- To establish the effect of acute arterial inflammation on vasomotor and fibrinolytic function in patients with stable coronary disease established on secondary preventative medications.

In patients with CHD and age-matched healthy volunteers

- To evaluate the tolerability of an intravenous bolus and infusion of the PDE 5 inhibitor sildenafil citrate.
- To determine whether acute treatment with sildenafil citrate has favourable effects on vascular tone and endogenous fibrinolytic function.

CHAPTER 2

METHODOLOGY:

**MEASUREMENT OF FOREARM BLOOD FLOW, PLASMA
FIBRINOLYTIC PARAMETERS, INFLAMMATORY CYTOKINES, TISSUE
PLASMINOGEN ACTIVATOR GENOTYPE AND ESTIMATION OF
EVENT FREE SURVIVAL**

2.1 INTRODUCTION

Endothelial function may be assessed *in vivo* by measuring the response to vaso-active substances released by, or those that interact with, the vascular endothelium. This approach has the advantage that vessels are studied in their physiological environment under the influence of neuronal, circulating, and local mediators [Benjamin *et al* 1995]. Local intra-arterial drug infusion permits the direct assessment of vascular responses without invoking concomitant effects on other organs.

2.1.1 ASSESSING VASCULAR FUNCTION

Although measurement of coronary responses may be of greatest clinical relevance, invasive coronary studies can only really be performed in patients undergoing angiography. The close correlation between coronary and peripheral endothelium-dependent responses [Anderson *et al* 1995] does, however, suggest that endothelial dysfunction may be a systemic state or that circulating factors have parallel effects in coronary and peripheral arteries [Vita 2005].

2.1.2 VENOUS OCCLUSION PLETHYSMOGRAPHY

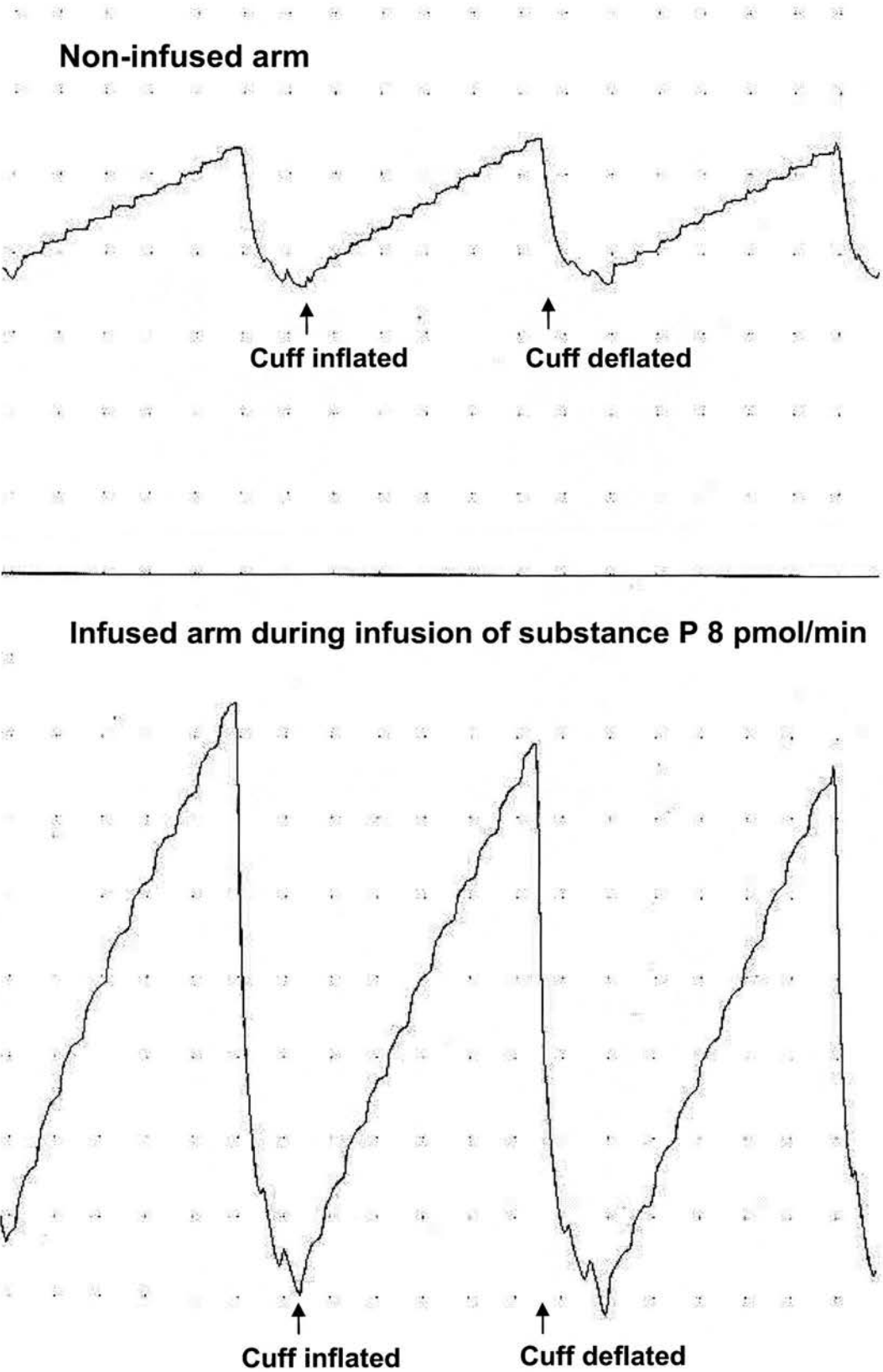
Methods of assessing resistance vessel function in the forearm are based on the principle of strain gauge venous impedance plethysmography. This technique examines the change in FBF during intra-arterial (brachial artery) administration of agonists at subsystemic, locally active doses (Figure 2.1) [Benjamin *et al* 1995; Webb 1995b]. The technique of venous occlusion plethysmography relies on

intermittently preventing venous drainage from the arm using upper arm cuffs inflated to above venous pressure whilst arterial inflow is unaltered: blood can enter the forearm but cannot escape. This results in a linear increase in forearm volume over time, which is proportional to arterial blood inflow (Figure 2.2). Under resting conditions, approximately 70% of total FBF is through skeletal muscle. As the hand contains a high proportion of arteriovenous shunts with a different pharmacology and physiology it is excluded from the circulation by the application of inflation cuffs at suprasystolic pressure during FBF measurement. The technique of bilateral FBF measurement is highly reproducible within individuals [Walker *et al* 2001] and is ideally suited to assessment of interventional strategies with repeated measurements [Wilkinson and Webb 2001].

Figure 2.1 Intra-brachial artery infusion system along with venous occlusion (upper arm) and suprasystolic pressure (wrist) cuffs in a left-handed (right arm, non-dominant) volunteer. A venous cannula for blood sampling is sited in the antecubital vein.



Figure 2.2 Typical bilateral blood flow recording during forearm plethysmography study.



2.2 GENERAL

2.2.1 ETHICAL CONSIDERATIONS

All studies were undertaken in accordance with the regulations of the Lothian Research Ethics Committee and with the Declaration of Helsinki of the World Medical Association. The written informed consent of each subject or patient was obtained before entry into the study.

2.2.2 SUBJECT RECRUITMENT

2.2.2.1 Subjects with Coronary Heart Disease

Potential subjects were identified from a database of patients undergoing coronary angiography within the Royal Infirmary of Edinburgh. Study information was posted to suitable subjects meeting study criteria defined below. The general practitioner and supervising hospital consultant were informed of all subjects who subsequently participated in the research study.

2.2.2.2 Inclusion and Exclusion Criteria

We recruited patients aged >18 years with angiographically proven CHD defined as $\geq 50\%$ luminal stenosis of at least one major epicardial coronary vessel or a history of MI (confirmed by a serial rise in creatine kinase of twice the upper limit of the normal reference range and the development of pathological Q-waves in at least two contiguous leads of the electrocardiogram). All patients had stable anginal symptoms and had not undergone coronary revascularisation within the preceding 3 months.

Exclusion criteria were significant cardiac failure, renal or hepatic impairment, systolic blood pressure <100 or >190 mmHg, diabetes mellitus, the regular use of non-steroidal anti-inflammatory medications (excluding aspirin 75 mg/d) or inability to rest supine during the forearm study.

2.2.2.3 Healthy Volunteer Subjects

A cohort of healthy control men who were age-matched to male subjects with CHD were also recruited. All control subjects were healthy normotensive euglycaemic non-smokers without any history of cardiorespiratory or vascular disease and were not taking any regular medications.

2.2.3 SUBJECT PREPARATION

Subjects were requested to abstain from alcohol for 24 hours and food, caffeine-containing drinks and tobacco for at least 4 hours before each study. Cardio-active medications were withheld on the morning of each CHD subject study visit. All studies were conducted in a quiet temperature-controlled room maintained at 22-25°C.

2.2.4 BLOOD PRESSURE MEASUREMENT

Blood pressure was monitored in the non-infused arm at intervals throughout each study using a semi-automated non-invasive oscillometric sphygmomanometer (Phillips, Agilent V24). Mean arterial pressure (MAP) was defined as the diastolic pressure plus a third of the pulse pressure.

2.3 VENOUS OCCLUSION PLETHYSMOGRAPHY

2.3.1 BRACHIAL ARTERY CANNULATION

The brachial artery of the non-dominant arm was cannulated with a 27-G steel needle (Cooper's Needle Works Ltd, Birmingham, UK) under 1% lidocaine (Xylocaine; Astra Pharmaceuticals Ltd, Kings Langley, UK) local anaesthesia. The cannula was attached to a 16-G epidural catheter (Portex Ltd, Hythe, UK) and patency maintained by infusion of saline (0.9%: Baxter Healthcare Ltd, Thetford, UK) via an IVAC P6000 syringe pump (IVAC Ltd, Basingstoke, UK). The total rate of intra-arterial infusions was maintained constant throughout all studies at 1 mL/min.

2.3.2 BLOOD FLOW MEASUREMENT

Blood flow was measured in the infused and non-infused forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges that were applied to the widest part of the forearm [Benjamin *et al* 1995]. Both arms were placed above the level of the right atrium and upper arm cuffs were inflated intermittently to 40 mmHg (venous occlusion) pressure for 10 seconds in every 15 seconds to achieve venous occlusion and obtain plethysmographic recordings. During measurement periods, the hands were excluded from the circulation by rapid inflation of the wrist cuffs to a pressure of 220 mmHg (suprasystolic) using E20 Rapid Cuff Inflators (D.E. Hokanson Inc., Washington, USA). Analogue voltage output from an EC-4 strain gauge plethysmograph (D.E. Hokanson Inc.) was processed by a PowerLab[®] analogue-to-digital converter and Chart[™] v5.0.1 software (AD Instruments Ltd,

Oxfordshire, UK) and recorded onto a Dell Latitude® laptop (Dell Computers Ltd, UK). Calibration was achieved using the internal standard of the plethysmograph.

2.3.3 PLETHYSMOGRAPHIC DATA ANALYSIS

Plethysmographic data were extracted from the Chart™ data files and FBFs were calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel 2002; Microsoft Corporation, USA). Recordings from the first 60 seconds after wrist cuff inflation were not used because of the variability in blood flow that this incurs [Benjamin *et al* 1995]. Usually, the last five flow recordings in each 3 minute measurement period were calculated and averaged for each arm.

2.3.4 REPRODUCIBILITY OF PLETHYSMOGRAPHIC DATA

Analysis of all data collected during the forearm plethysmography study was undertaken by a single operator in a blinded fashion as appropriate. Forearm blood flow responses are reported as absolute blood flow responses (mL/100 mL tissue/min) in the infused and non-infused arm unless stated otherwise. Previous work has demonstrated intra-subject variability and coefficient of variation of 7.8% and 27% for FBF across resting conditions and the dose range of vaso-agonists used [Roberts *et al* 1986; Altenkirch *et al* 1990; Walker *et al* 2001; Newby *et al* 2002].

2.4 VENOUS SAMPLING AND LABORATORY ASSAYS

2.4.1 FOREARM VENOUS SAMPLING

Following administration of local anaesthetic, venous cannulae (17-G) were inserted into large subcutaneous veins of the antecubital fossa in both arms as described previously [Newby *et al* 1997]. Blood was withdrawn simultaneously from each arm and collected into acidified buffered citrate (Biopool® Stabilyte™, Umeå, Sweden; t-PA), trisodium citrate (Monovette®, Sarstedt, Nümbrecht, Germany; PAI-1 and prothrombin F1+2) potassium ethylene diamine tetraacetic acid (Monovette®, Sarstedt, Nümbrecht, Germany; cotinine; cytokines; full blood count) and serum gel (Monovette®, Sarstedt, Nümbrecht, Germany; CRP; clinical chemistry) tubes.

2.4.2 SAMPLE PREPARATION

Citrate and acidified buffered citrate samples were centrifuged at 2,000 g for 30 minutes at 4°C, EDTA samples at 1000g for 10 minutes at 20°C. Serum samples were centrifuged at 2,000 g for 20 minutes after being allowed to clot on ice. Platelet free plasma or serum was decanted and stored at -80°C before assay [Kluft and Verheijen 1990].

2.4.3 PLASMA FIBRINOLYTIC AND HAEMOSTATIC ASSAYS

Plasma t-PA and PAI-1 antigen concentrations were determined using an enzyme-linked immunosorbent assay; Coaliza® t-PA [Booth *et al* 1987] and Coaliza® PAI-1 (Chromogenix AB, Mölndal, Sweden) [Declerck *et al* 1988] respectively. Plasma t-PA activity was determined by a photometric method, Coatest® t-PA (Chromogenix

AB) [Gram *et al* 1987]. Intra-assay coefficients of variation are 7.0% and 5.5% for t-PA and PAI-1 antigen, and 4.0% for t-PA activity, with inter-assay coefficients of variability of 4.0%, 7.3% and 4.0% respectively [Newby *et al* 2002]. The sensitivities of the assays are 0.5 ng/mL, 2.5 ng/mL and 0.10 IU/mL respectively [Newby *et al* 2002]. Prothrombin fragment 1 and 2 (F1+2; Enzygnost F1+2; Dade Behring, Milton Keynes, UK), was determined using an enzyme-linked immunosorbent assay with intra- and inter- coefficients of variation of 10.4% and 11% respectively [Pelzer *et al* 1991]. All coagulation assays were performed in duplicate and the mean value taken.

2.4.4 CYTOKINE AND C-REACTIVE PROTEIN ASSAYS

Plasma TNF- α (Quantikine R&D systems Inc., Minneapolis, USA) and IL-6 (Dako A/S, Glostrup, Denmark) concentrations were determined using enzyme-linked immunosorbent assays [Chia *et al* 2003a; 2003b]. Intra-assay, and inter-assay coefficients of variability were 5.2% and 7.2% and 4.2% and 6.4% for plasma TNF- α and IL-6 respectively. Serum C-reactive protein concentrations were determined using with a validated highly sensitive assay (*hs*-CRP, Department of Clinical Biochemistry; Fife NHS Trust, UK) [Roberts *et al* 2001] using the method of particle-enhanced immunonephelometry (Behring BN II nephelometer, Dade Behring Inc.). Intra-assay and inter-assay coefficients of variability for *hs*-CRP were 3.7% and 4.2% respectively.

2.4.5 HAEMATOCRIT AND BIOCHEMICAL ASSAYS

Total white cell count and haematocrit and biochemical assays were undertaken on stored venous samples (Departments of Haematology and Clinical Biochemistry, Lothian NHS University Hospitals Trust, UK). Plasma cotinine concentrations were determined using high performance liquid chromatography (ABS Laboratories Ltd; London, UK) [Feyerabend and Russell 1990]. A plasma value of greater than 13.7 ng/mL yields a 100% specificity and 96% sensitivity for smoking and non-smoking status with a detection limit of 0.1 ng/mL [Feyerabend and Russell 1990].

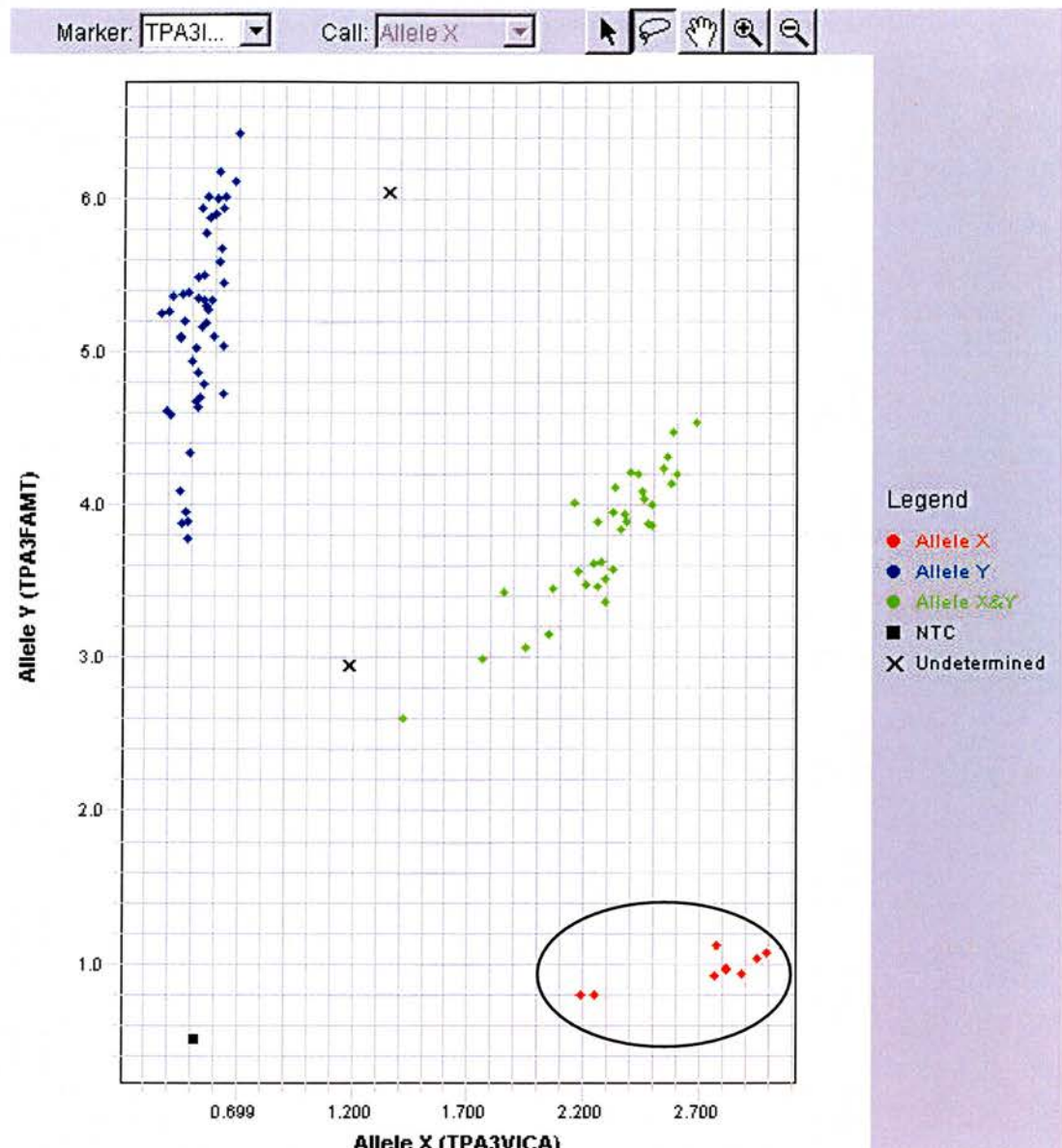
2.5 GENOTYPING

2.5.1 DNA EXTRACTION AND POLYMERASE CHAIN REACTION ASSAY

All genetic work was undertaken by the Wellcome Trust Clinical Research Facility (WTCRF) Genetics Core Laboratory (Western General Hospital, Edinburgh, UK). Genomic DNA was isolated from nucleated cells from frozen whole blood. Genotyping was performed by allele discrimination analysis using the 5' nuclease assay [Livak *et al* 1995]. In this assay, the polymorphic target sequence is amplified by PCR in the presence of two fluorogenic probes specific for each allele. Probes hybridise to their target during the annealing step and the reporter dye is cleaved by the 5' nuclease activity of *Taq* polymerase which gives rise to an increased fluorescent signal. Any mismatch greatly reduces the efficiency of probe hybridisation and cleavage allowing the allelic content of a sample to be determined by comparing the fluorescent contribution of each dye (Figure 2.3).

Figure 2.3 Allelic discrimination scatterplot for t-PA 27,445 T→A (intron 10) polymorphism showing PCR products obtained from AA homozygotes (circled, lower right), heterozygotes (middle right) and TT homozygotes (top left) (courtesy of Angie Fawkes, WTCRF Genetics Core).

t-PA - tissue plasminogen activator; PCR - polymerase chain reaction; WTCRF - Wellcome Trust Clinical Research Facility.



2.5.2 TISSUE PLASMINOGEN ACTIVATOR POLYMORPHISMS

For the -7,351 C→T (upstream enhancer), 20,099 T→C (exon 6), and 27,445 T→A (intron 10) polymorphisms genotyping was performed by allele discrimination analysis using the 5' nuclease assay. Oligonucleotide primers and fluorescently labelled probes were obtained from Applied Biosystems (Applied Biosystems, Foster City, California, USA) on the basis of previously published DNA and primer sequences [Ladenvall *et al* 2000; Ladenvall *et al* 2002]. Polymorphic target sequences were amplified by PCR using two allelic specific probes. Real time quantitative PCR was performed in an ABI PRISM[®] 7900HT Sequence Detector System (Applied Biosystems, California, USA). Thermal cycling conditions were two initial holds (50° C for 2 minutes and 95° C for 10 minutes) followed by a 40-cycle two-step program (95° C for 15 seconds and 60° C for 1 minute) utilising a standardised operating protocol (WTCRF Genetics Core; <http://www.wtcrf.ed.ac.uk>). The fluorescent contribution of each dye was determined directly after the completion of PCR.

For the *Alu*-intronic polymorphism, the insertion specific sequence was submitted to the Applied Biosystems Assay-by-design service. Gene expression assays consisted of unlabelled PCR primers and TaqMan[®] MGB probes (FAM dye labelled). Forward and reverse primers were 5'-CGTAACAGGACAGCTCACAGTT-3 and 5'-CCCTAGGAGAACTTCTCTTTAACTTTGTT-3 respectively. The insertion specific probe was labelled in its 5' end with FAM and in its 3' end with a NFQ (non fluorescent quencher): FAM- ACCCTATGAGATTAGAACAC-NFQ. Real time quantitative PCR was performed with a ABI PRISM[®] 7900HT Sequence Detector

using the standardised operating protocol described (WTCRF Genetics Core; <http://www.wtcrf.ed.ac.uk>). *Alu*-insertion PCR products were normalised to threshold values against an endogenous control (single copy *RNase P* gene). Each sample was run in triplicate with both the gene of interest and the *RNase P* control. Genotype for the *Alu*-intronic polymorphism was calculated from differences in the threshold cycle between the *RNase P* and *Alu*-Ins sequence.

A confirmatory assay of *Alu*-Ins/Del genotypes utilised PCR amplification of the Ins fragment with primers designed to flank the region of interest (PLAT.1 5' to 3' GTGAAAAGCAAGGTCTACCAG and PLAT.2 5' to 3' GACACCGAGTTCATCTTGAC). PCR was performed on a DNA engine tetrad from MJ Research (Bio-Rad Laboratories, Inc., California, USA). Thermal cycling conditions were 94° C for 2 minutes followed by 35-cycles (30 seconds at 94° C for and 30 seconds at 60° C and 30 seconds at 72° C) with a final extension step of 10 minutes at 72° C. A 10 µL sample of this reaction was analysed on a 2.5% agarose gel and following electrophoresis DNA was visualised with ethidium bromide and UV transillumination. Amplification of the products produces a 570-bp fragment from chromosomes with the Ins allele and a 260-bp fragment from those without (Del).

2.6 FREQUENCY OF CARDIOVASCULAR EVENTS DURING FOLLOW-UP

The frequency of acute thrombotic-related patient events, defined as death from a cardiovascular cause, MI, ischaemic stroke (CVA), and emergency hospitalisation for myocardial ischaemia, were determined through the Information and Statistics Division of the National Health Service in Scotland and the General Register Office in Scotland. These systems have been successfully applied to evaluate cardiovascular morbidity and mortality for subjects resident within Scotland [Fox *et al* 2002].

2.7 DATA ANALYSIS AND STATISTICS

Data were examined, where appropriate, by analysis of variance (ANOVA) with repeated measures, two-tailed Student's *t*-test and Chi-squared test using GraphPad Prism (GraphPad Software, California, USA). Results are expressed as mean and standard error of mean (SEM) unless otherwise stated. Statistical significance was assigned at the 5% level.

2.7.1 CALCULATION OF NET T-PA RELEASE

For the forearm studies, estimated net release of t-PA activity and antigen was defined as the product of the infused forearm plasma flow (based on the haematocrit, Hct and the infused FBF) and the concentration difference between the infused ($[t\text{-PA}]_{\text{Inf}}$) and non-infused arms ($[t\text{-PA}]_{\text{Non-inf}}$).

$$\text{Estimated net forearm t-PA release} = \text{FBF} \times \{1 - \text{Hct}\} \times \{[\text{t-PA}]_{\text{Inf}} - [\text{t-PA}]_{\text{Non-inf}}\}$$

Area under the curves (AUCs) for the dose-response of t-PA release were calculated using the trapezoid rule [Matthews *et al* 1990].

2.7.2 TISSUE PLASMINOGEN ACTIVATOR GENOTYPE

Allelic discrimination for the polymorphisms of interest were ascertained by an individual without information on case status or vascular study data. Allele frequencies were estimated by gene counting. Chi-square test was used to evaluate deviations of genotype distributions from the Hardy-Weinberg equilibrium. The linkage disequilibrium coefficient D' was calculated as previously described [Devlin and Risch 1995].

2.7.3 CARDIOVASCULAR EVENT RATES

Cumulative event rates were estimated by Kaplan-Meier survival curves, and probability values were determined with the log-rank test. In those patients who experienced multiple events, survival analysis was restricted to the first event. For Kaplan-Meier analyses, subjects were divided into quartiles according to estimated net release of t-PA antigen.

CHAPTER 3

ENDOTHELIAL FIBRINOLYTIC CAPACITY PREDICTS FUTURE ADVERSE CARDIOVASCULAR EVENTS IN PATIENTS WITH CORONARY HEART DISEASE

Robinson SD, Ludlam CA, Boon NA, Newby DE.
Endothelial fibrinolytic capacity predicts future adverse
cardiovascular events in patients with coronary heart disease.
(Arterioscler Thromb Vasc Biol, in print)

3.1 SUMMARY

The endothelium-derived fibrinolytic factor t-PA is a major determinant of vessel patency following coronary plaque rupture and thrombosis. We assessed whether endothelial fibrinolytic capacity predicts atherothrombotic events in patients with CHD. Plasma t-PA and PAI-1 concentrations, and net t-PA release were measured during intra-brachial substance P infusion in 97 patients with angiographically proven stable CHD. Forearm blood flow was measured during infusion of substance P and sodium nitroprusside. Cardiovascular events (cardiovascular death, MI, CVA and emergency hospitalisation for myocardial ischaemia) were determined during a median follow-up of 34 months. Substance P caused a dose-dependent increase in plasma t-PA ($p<0.001$) but not PAI-1 ($p=ns$) concentrations. Patients experiencing a cardiovascular event ($n=20$) during follow-up had similar baseline characteristics to those free of events ($p=ns$ for all). However, net t-PA release was 91% lower in the patients who experienced death, MI or CVA, and 44% lower in those who suffered death, MI, CVA or hospitalisation for myocardial ischaemia ($p\leq 0.02$). Major adverse cardiovascular events were most frequent in those with the lowest fibrinolytic capacity ($p=0.04$ for trend); the patients with the lowest quartile of t-PA release had the highest rate of adverse events ($p=0.02$). Endothelial fibrinolytic capacity, as measured by stimulated t-PA release, predicts the future risk of adverse cardiovascular events in patients with CHD. We suggest that endothelial fibrinolytic capacity is a powerful novel determinant of cardiovascular risk.

3.2 INTRODUCTION

The endogenous fibrinolytic system protects the circulation from intravascular fibrin formation and thrombosis. In the presence of developing thrombus, the fibrinolytic factor t-PA is rapidly released from the vascular endothelium by the coagulation factors thrombin and factor Xa [Emeis 1992]. Once released, t-PA increases the enzymatic conversion of plasminogen to plasmin, a process increased a 1,000-fold by the co-localisation of t-PA and plasminogen in the presence of fibrin [Ranby 1982; van Zonneveld *et al* 1986], thereby ensuring plasmin activation, local fibrin degradation and thrombus dissolution are tightly regulated to sites of fibrin deposition which characterise arterial injury. The rapidity and extent of acute t-PA release from the endothelium is therefore a critical factor in determining the efficacy of local endogenous fibrinolysis [Hrafnkelsdottir *et al* 2004a; Oliver *et al* 2005].

Areas of endothelial denudation and thrombus deposition are a common finding on the surface of atheromatous plaques and are often subclinical [Mann and Davies 1999]. Through t-PA release, endogenous fibrinolysis is usually able to prevent thrombus propagation, vessel occlusion and tissue infarction, although organisation of the residual thrombus may lead to plaque growth and expansion [Mann and Davies 1999]. The resolution of thrombus following atheromatous plaque rupture, and the resulting clinical sequelae, may therefore be critically dependent on the efficacy of endogenous fibrinolysis [Rosenberg and Aird 1999]. Accordingly the capacity of the endothelium to release t-PA may predict the outcome of individual plaque events and long-term cardiovascular risk.

Intra-arterial infusion of methacholine [Jern *et al* 1994], substance P [Newby *et al* 1997] and bradykinin [Brown *et al* 1999] cause a rapid and sustained release of t-PA from the endothelium and acute stimulated t-PA release has been measured within the forearm [Jern *et al* 1994; Newby *et al* 1997] and coronary [Minai *et al* 2001; Newby *et al* 2001] circulations of man. We have previously demonstrated impaired forearm t-PA release in cigarette smokers [Newby *et al* 1999], a group who are at particular risk of coronary thrombosis and MI [Burke *et al* 1997]. Although this suggests that endogenous fibrinolysis plays an important role in the pathogenesis of coronary thrombosis, the relationship between the capacity to release t-PA and the future risk of adverse cardiovascular events is unknown. The aim of the present study was, therefore, to determine whether endothelial fibrinolytic capacity predicts the future risk of atherothrombotic events in patients with stable CHD.

3.3 METHODS

3.3.1 PATIENTS

We recruited patients with CHD defined as $\geq 50\%$ luminal stenosis of at least one major epicardial coronary vessel. All patients had stable anginal symptoms and had not undergone coronary revascularisation within the preceding 3 months. Exclusion criteria were significant cardiac failure, renal impairment, systolic blood pressure <100 or >190 mmHg, diabetes mellitus or the regular use of non-steroidal anti-inflammatory medications (excluding aspirin 75 mg/d). All studies were undertaken with the approval of the local Research Ethics Committee, the written informed consent of each subject, and in accordance with the Declaration of Helsinki.

3.3.2 DRUGS

Substance P (Clinalfa AG, Switzerland) and sodium nitroprusside (David Bull Laboratories, UK) were administered following dissolution in 0.9% saline. All solutions were freshly prepared on the day of study.

3.3.3 FOREARM STUDY PREPARATION

Subjects abstained from alcohol for 24 hours and from food, tobacco and caffeine-containing drinks for at least 4 hours before each study visit. Cardio-active medications were withheld on the morning of study. All studies were carried out in a quiet temperature-controlled room maintained at 22-25°C. Patients rested recumbent, strain gauges and cuffs were applied. Venous cannulae were inserted into each arm

and the brachial artery of the non-dominant arm cannulated. Blood pressure and heart rate were measured using a semi-automated non-invasive sphygmomanometer.

3.3.4 STUDY DESIGN

Following a 30 minute saline infusion, intra-arterial substance P at 2, 4 and 8 pmol/min and sodium nitroprusside at 2, 4 and 8 µg/min were administered in a randomised order with a 15 minute saline washout period between each agent. Forearm blood flow was measured at baseline and with each dose of vasodilator. Venous blood sampling for plasma t-PA and PAI-1 antigen concentration estimation was undertaken before and during each dose of substance P.

3.3.5 LONG-TERM FOLLOW-UP

Atherothrombotic cardiovascular events, defined as death from cardiovascular causes, MI, CVA, and emergency hospitalisation for myocardial ischaemia, were determined through the Information and Statistics Division of the National Health Service in Scotland and the General Register Office in Scotland. These databases combine high quality data and consistency, with national coverage and the ability to link data to allow patient based analysis and follow-up. These systems have been successfully applied to evaluate cardiovascular morbidity and mortality for subjects resident within Scotland [Fox *et al* 2002].

3.3.6 STATISTICAL ANALYSIS

Data of baseline characteristics are expressed as mean \pm standard deviation (SD) or frequency (n, %) and compared using two-tailed Student's *t*-test and Fisher's exact

test where appropriate. Responses to substance P and sodium nitroprusside were examined by ANOVA for repeated measures. To reduce the variability of blood flow data between groups [Benjamin *et al* 1995; Webb 1995b], FBF responses to the vasodilators were calculated as the ratio between the infused and control arms expressed as a percentage of the ratio measured during the baseline control period as follows:

$$\% \text{ Change in blood flow} = 100 \times \{I_t/NI_t - I_b/NI_b\} / I_b/NI_b$$

where I_b and NI_b are the infused and non-infused FBFs at baseline (time 0) respectively, and I_t and NI_t are the infused and non-infused FBFs at a given time point respectively.

Estimated net release of t-PA was defined as the product of the infused forearm plasma flow, and the concentration difference between the infused and non-infused forearms [Newby *et al* 1997; Newby *et al* 1999; Oliver *et al* 2005]. Cumulative event rates were estimated by Kaplan-Meier survival curves, and probability values were determined with the log-rank test. In those patients who experienced multiple events, survival analysis was restricted to the first event. For Kaplan-Meier analyses, subjects were divided into quartiles according to estimated net release of t-PA antigen. All statistical analysis was completed using GraphPad Prism (GraphPad Software, California, USA) with statistical significance being assigned at the 5% level.

3.4 RESULTS

Endothelial fibrinolytic capacity was measured in 97 subjects with angiographically proven CHD who were followed-up for a median of 34 months (range 27 to 45 months). Over the follow-up period, two patients died from cardiovascular disease, two suffered an MI, two had a CVA and 14 had an emergency hospital admission for myocardial ischaemia. Patients experiencing cardiovascular events had similar baseline characteristics and use of secondary preventative medications to those free from events ($p=ns$ for all; Table 3.1).

Table 3.1 Baseline subject characteristics

	All Patients (n=97)	Subjects with events (n=19)	Subjects without events (n=78)	P value*
Age, years	59 ± 7	62 ± 8	59 ± 7	0.10
Sex, male/female	79/18	16/3	63/15	1.00
BMI, kg/m²	29 ± 4	28 ± 4	29 ± 4	0.36
Systolic BP, mmHg	132 ± 19	135 ± 20	132 ± 19	0.53
Diastolic BP, mmHg	76 ± 12	75 ± 11	76 ± 13	0.88
Pulse, bpm	57 ± 9	57 ± 10	57 ± 9	0.93
Cigarette smoker, (%)	31	37	29	0.58
Co-morbidity, (%)				
Previous MI	41	53	38	0.30
Hypertension	48	47	49	1.00
FHx CHD	33	47	29	0.18
Symptomatic CVD	4	11	3	0.17
Extent of coronary disease, (%)				
1 vessel	45	42	46	0.75
2 vessels	27	32	26	0.60
3 vessels	28	26	28	0.92
Medical Therapy, (%)				
Aspirin	99	100	99	0.62
Lipid lowering therapy	93	95	93	0.71
ACE inhibitor, ARB	31	32	31	0.94
Urea, mmol/L	5.6 ± 1.3	5.4 ± 1.4	5.6 ± 1.3	0.60
Creatinine, µmol/L	94 ± 13	98 ± 15	92 ± 12	0.11
Glucose, mmol/L	5.7 ± 0.9	6.0 ± 1.0	5.6 ± 0.8	0.07
Total chol, mmol/L	4.5 ± 1.0	4.6 ± 1.1	4.5 ± 0.9	0.62
LDL chol, mmol/L	2.6 ± 0.8	2.6 ± 1.1	2.5 ± 0.6	0.66
t-PA antigen, ng/mL	9.7 ± 3.9	9.9 ± 5.1	9.7 ± 3.5	0.84
PAI-1 antigen, ng/mL	43.7±24.5	33.9±18.6	45.9±25.2	0.07

Values are mean ± SD

*Unpaired *t*-test or Fishers exact test for subjects with, versus without, events (death from a cardiovascular cause, MI, CVA, emergency hospitalisation for myocardial ischaemia).

BMI – body mass index; bpm – beats per minute; MI – myocardial infarction; FHx – family history; CHD – coronary heart disease; CVD – cerebrovascular disease; ACE – angiotensin-converting enzyme; ARB – angiotensin receptor blocker; chol – cholesterol; t-PA – tissue plasminogen activator; PAI-1 – plasminogen activator inhibitor type-1.

Substance P caused a dose-dependent increase in plasma t-PA ($p<0.001$, ANOVA) but not PAI-1 ($p=ns$) concentrations. The concentration differences of t-PA antigen between the forearms and estimated net release of t-PA both increased dose dependently ($p<0.001$ for both, ANOVA) although this increase was reduced in subjects who subsequently experienced cardiovascular events ($p\leq 0.02$, ANOVA; Figure 3.1). Specifically, net release of t-PA during substance P infusion was 91% lower in those subjects suffering cardiovascular death, MI or stroke ($p=0.01$, ANOVA), and 44% lower in those with cardiovascular death, MI, stroke or emergency hospitalisation for myocardial ischaemia ($p=0.02$, ANOVA). Major adverse cardiovascular events were most frequent in those with the lowest fibrinolytic capacity ($p=0.04$ for trend); patients with the lowest quartile of t-PA release had the highest rate of adverse events ($p=0.02$; Figure 3.2).

Figure 3.1 Concentration differences between the forearms of plasma t-PA antigen (lower panels), and estimated net release of t-PA antigen (upper panels), in subjects with (●), and without (○), death, MI or stroke (left hand panels) or death, MI, stroke or emergency hospitalisation for myocardial ischaemia (right hand panels).
 * $p=0.01$, † $p=0.008$ and ‡ $p=0.02$, ANOVA (●) versus (○); t-PA – tissue plasminogen activator; MI – myocardial infarction; ANOVA – analysis of variance.

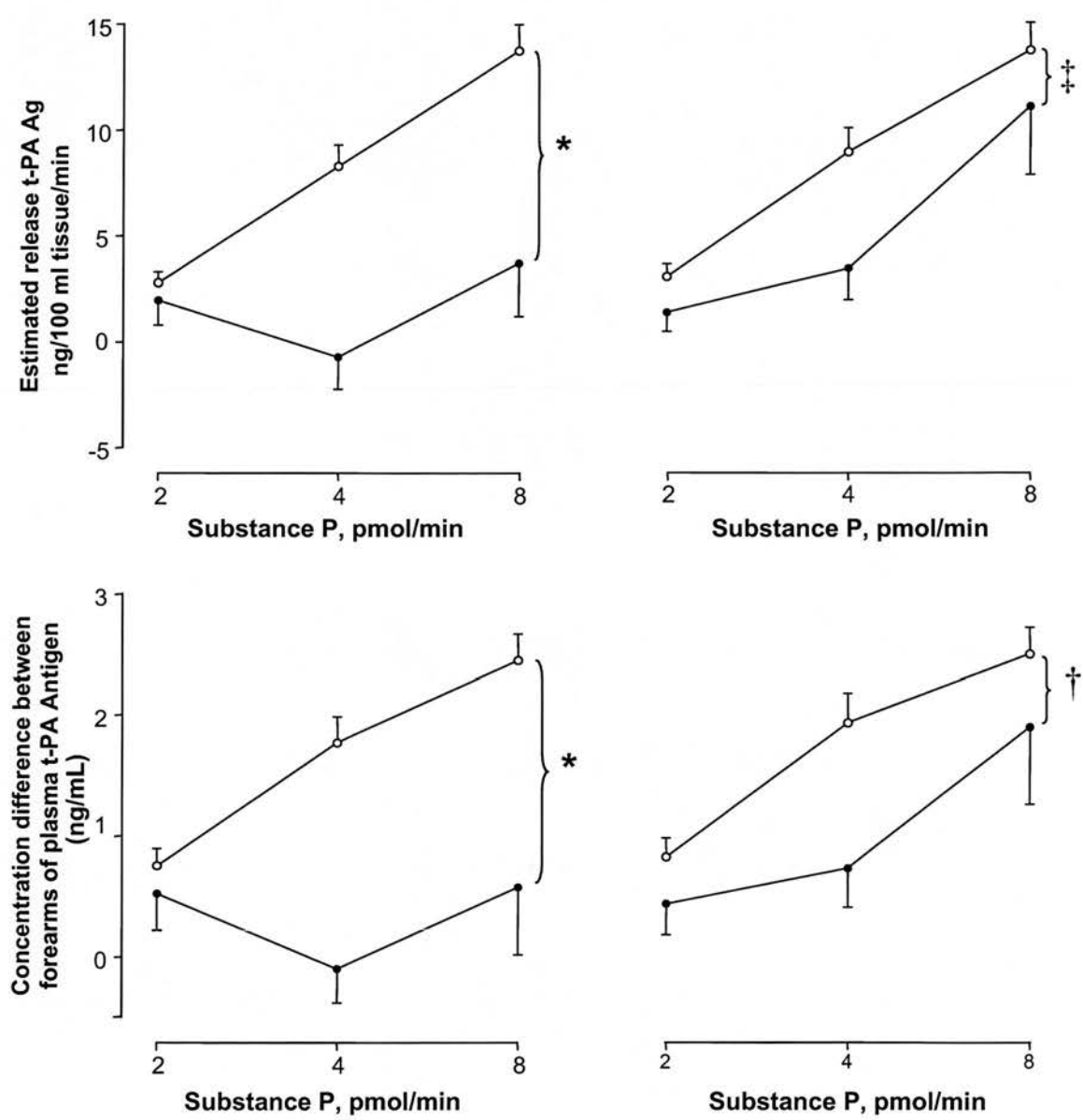
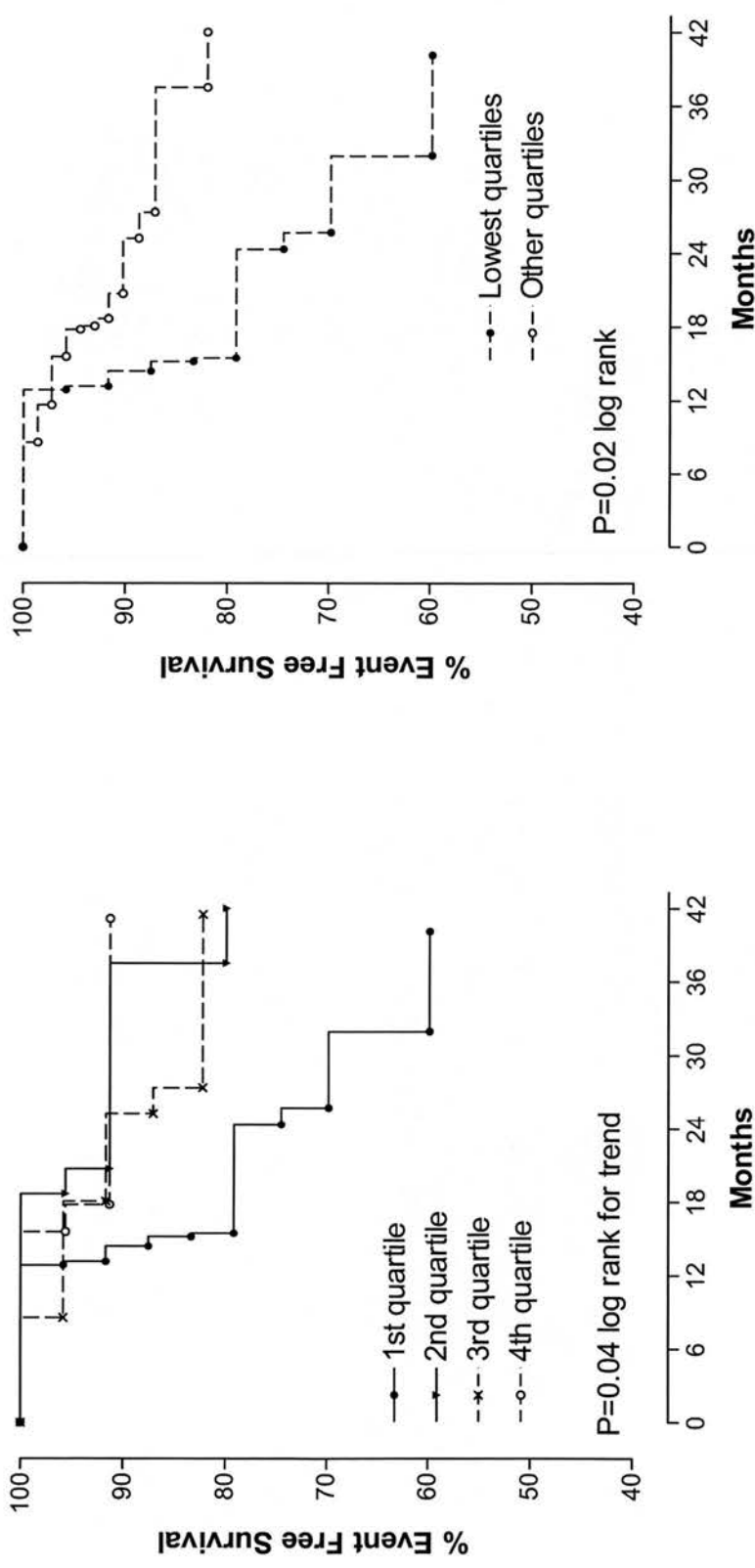
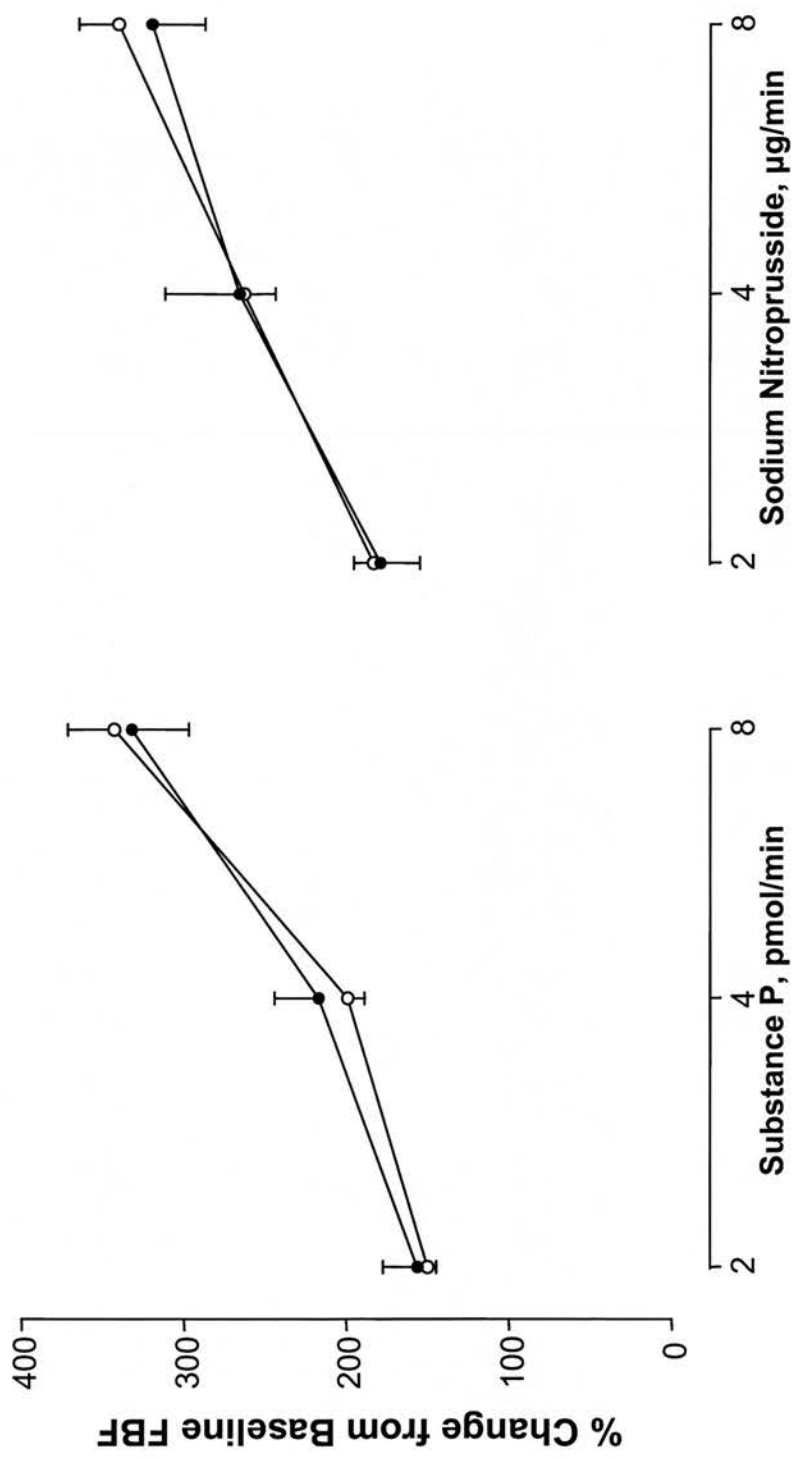


Figure 3.2 Cumulative proportion of patients without cardiovascular events during follow-up (Kaplan-Meier). Subjects are divided into quartiles for estimated net release of t-PA antigen. $p=0.04$, log-rank across quartiles; $p=0.02$, lowest versus upper quartiles; t-PA – tissue plasminogen activator.



There was no difference in resting FBF between those subjects with and without atherothrombotic events (2.8 ± 0.2 mL/100 mL tissue/min *versus* 2.7 ± 0.1 mL/100 mL tissue/min respectively, $p=\text{ns}$). Substance P and sodium nitroprusside caused dose-dependent increases in infused FBF in all subjects ($p<0.001$, ANOVA). There were no differences in either endothelium-dependent or -independent blood flow responses between subjects in either group (Figure 3.3).

Figure 3.3 Forearm blood flow (FBF) during incremental doses of substance P (left), sodium nitroprusside (right) in subjects with (●), and without (○), death, myocardial infarction, stroke or emergency hospitalisation for myocardial ischaemia. $p<0.001$, ANOVA dose response, $p=ns$ for both, (●) versus (○).



3.5 DISCUSSION

This is the first study to assess the relationship between endothelial fibrinolytic capacity and the future risk of atherothrombotic cardiovascular events. The population that we chose to study was a homogenous cohort of patients with stable CHD and a typical distribution of conventional cardiovascular risk factors. Even though this was a relatively low risk population receiving optimal secondary preventative therapy, endothelial fibrinolytic capacity has emerged as a powerful novel determinant of future cardiovascular risk.

In a meta-analysis of prospective observational studies [Lowe *et al* 2004], the risk of CHD is increased in subjects with plasma t-PA antigen concentrations in the *highest* tertile compared to those in the *lowest* tertile. This may seem counterintuitive but in part reflects the concomitant rise of plasma PAI-1 concentrations and the associated reduction in t-PA activity. Moreover, in the basal state, the endogenous fibrinolysis system is effectively inactive and does not influence *in situ* thrombus formation. Neither basal plasma t-PA nor PAI-1 concentrations control the local vascular fibrinolytic capacity which is determined by the acute release of t-PA from the endothelium [Hrafnskeldottir *et al* 2004a]. This underscores the importance of assessing the pathophysiologically relevant measure of acute stimulated endothelial t-PA release.

We and others have demonstrated that cigarette smoking [Newby *et al* 1999; Newby *et al* 2001; Pretorius *et al* 2002], but not hypercholesterolaemia [Newby *et al* 2002],

is associated with a characteristic and substantial reduction in endothelial t-PA release. There is also a good correlation and consistency between the endothelial fibrinolytic capacity of the forearm [Newby *et al* 1999; Witherow *et al* 2002] and the coronary circulation [Minai *et al* 2001; Newby *et al* 2001]. The forearm vascular bed is relatively protected from the development of atheroma and it therefore seems likely that changes in its fibrinolytic capacity are indicative of a systemic effect and not simply local plaque burden. These considerations are, therefore, in keeping with the hypothesis that atherosclerosis is a systemic disorder, and that acute t-PA release should be considered a distinct marker of endothelial function.

Intravascular thrombus formation is a key feature of clinical atherosclerotic events associated with eroded or unstable coronary plaques. The importance of endogenous t-PA release is exemplified by the high rate of spontaneous reperfusion in the infarct-related artery after acute MI, occurring in up to 30% of patients within the first 12 hours [DeWood *et al* 1980; Armstrong *et al* 1989; Rentrop *et al* 1989]. Any reduction in the acute dynamic fibrinolytic response decreases the capacity to lyse intraluminal thrombus and the likelihood of restoring vessel patency. In this prospective observational cohort study, we have further demonstrated that the capacity to release t-PA appears to be a major determinant of the risk of cardiovascular events and suggests that endothelial fibrinolytic capacity has a crucial role in the pathogenesis of atherothrombosis.

Endothelial dysfunction is characterised by the disruption of multiple homeostatic pathways predisposing to vasoconstriction, platelet activation and thrombosis. To

date, most clinical studies on endothelial function have focused on endothelium-dependent vasomotion with decreased responses associated with an increased incidence of future adverse events [Suwaidi *et al* 2000; Heitzer *et al* 2001; Halcox *et al* 2002b]. Although a useful surrogate marker, the pathophysiological mechanism linking impaired endothelium-dependent vascular smooth muscle relaxation and future atherothrombotic events remains unclear. Moreover, the regulation of vessel tone may not be the facet of vascular function most closely allied to the future risk of atherothrombotic events. In this current study, we did not observe a difference in endothelium-dependent or –independent blood flow responses between subjects with, and without, subsequent cardiovascular events. This disparity may reflect a lack of power or the use of differing endothelium-dependent vasodilators. Thus far, studies assessing the prognostic value of endothelium-dependent vasodilatation have used acetylcholine. We chose to use substance P because this is a potent stimulant of endothelial t-PA release, and acetylcholine does not cause demonstrable t-PA release [Brown *et al* 1999]. Reports of preserved endothelium-dependent vasodilatation in smokers [Jacobs *et al* 1993] and in patients with hypertension [Cockcroft *et al* 1994a] despite reduced acute t-PA release [Hrafinkelsdottir *et al* 1998; Pretorius *et al* 2002] suggest that, in some circumstances, reduced t-PA release may be a more sensitive marker of endothelial dysfunction [Oliver *et al* 2005]. These data also highlight the complexity of vascular biology and demonstrate that endothelial dysfunction is not a single clinical entity encompassing a uniform pathophysiological response to vascular injury.

3.5.1 STUDY LIMITATIONS

Plaque growth is induced by episodic subclinical plaque disruption [Mann and Davies 1999] and if local t-PA release is impaired, the continued presence of thrombus may favour smooth muscle migration, the production of new connective tissue and plaque expansion [Davies 2000b]. In keeping with this hypothesis, genetic murine models of plasminogen deficiency [Carmeliet *et al* 1994; Xiao *et al* 1997] as well as PAI-1 overexpression [Eren *et al* 2002] have shown that reduced fibrinolytic potential is associated with enhanced macrovascular fibrin deposition and accelerated atherogenesis. As we do not have follow-up angiographic data, we do not know if those subjects with the lowest local t-PA release or recurrent cardiovascular events exhibited a greater progression of angiographic disease over the study period. We have previously demonstrated an inverse correlation between acute coronary t-PA release and local atheromatous plaque burden [Newby *et al* 2001] and prospective studies of coronary t-PA release and quantification of disease progression would be of interest.

In keeping with the risk factor profile and use of secondary preventative therapies in our study cohort, relatively few events occurred over the study period. Despite this, similar results were noted for outcomes regardless of whether emergency hospitalisation for myocardial ischaemia was included. Although the risk factor profile and severity of coronary artery disease were similar in those patients with or without events, the study population size was modest and we were therefore unable to explore potential interactions and independence of endothelial fibrinolytic capacity with conventional cardiovascular risk factors or the extent of coronary artery disease.

Confirmation of our findings in larger at risk cohorts with differing cardiovascular risk profiles is now warranted to explore the importance and more widespread relevance of endothelial fibrinolytic capacity.

In summary, t-PA release from the endothelium is a distinct marker of endothelial function. In patients with stable CHD, we have shown that a reduction in acute t-PA release predicts an increased risk of adverse cardiovascular events. Further studies of the factors modifying the endogenous fibrinolytic capacity have the potential to provide major new insights into the pathophysiology of CHD and to shape future therapeutic interventions.

CHAPTER 4

TISSUE PLASMINOGEN ACTIVATOR GENETIC POLYMORPHISMS DO NOT INFLUENCE TISSUE PLASMINOGEN ACTIVATOR RELEASE IN PATIENTS WITH CORONARY HEART DISEASE

Robinson SD, Ludlam CA, Boon NA, Newby DE.

Tissue plasminogen activator genetic polymorphisms do not influence
tissue plasminogen activator release in patients with coronary heart disease.

J Thromb Haemost 2006;**4**(10):2262-2269.

4.1 SUMMARY

Genetic factors influence plasma concentrations of a number of haemostatic and fibrinolytic factors including t-PA. Previous work has demonstrated a link between t-PA release and t-PA polymorphisms in healthy volunteers. We determined if polymorphisms of the t-PA gene influence acute endogenous t-PA release in patients with CHD. Forearm blood flow and plasma t-PA concentrations were measured in response to intra-brachial infusion of substance P and sodium nitroprusside in 96 patients with stable CHD. Genotyping was performed using a *Taqman* PCR assay specifically designed to detect the polymorphisms of interest: (i) *Alu*-repeat Ins/Del sequence, (ii) C→T substitution in an upstream enhancer region (-7,351 C/T), (iii) T→C in exon 6 (20,099 T/C), and (iv) T→A (27,445 T/A) in intron 10. Substance P and sodium nitroprusside caused dose-dependent increases in FBF in all patients ($p<0.001$ for all) that were independent of the four genetic polymorphisms. Similarly, there were no differences in basal plasma t-PA antigen concentrations or net t-PA release between genotypes. Compared to non-smokers, smokers exhibited impaired substance P-induced vasodilatation ($p<0.001$) and t-PA release ($p=0.05$). Despite confirming previous findings in cigarette smokers, we have found no effect of polymorphisms of the t-PA gene on two complementary aspects of endothelial function. We conclude that genetic variation at the t-PA locus is unlikely to have a major influence on acute t-PA release in subjects with established CHD.

4.2 INTRODUCTION

Coronary heart disease and its risk factors, such as cigarette smoking, hyperlipidaemia and hypertension, are associated with impaired endothelium-dependent vasorelaxation [Creager *et al* 1990; Celermajer *et al* 1993; Panza *et al* 1993] and t-PA release [Hrafnkelsdottir *et al* 1998; Newby *et al* 1999; Pretorius *et al* 2002]. These aspects of endothelial function are important since plasma fibrinolytic variables and endothelium-dependent vasodilatation independently predict future cardiovascular risk [Meade *et al* 1993; Suwaidi *et al* 2000].

Genetic factors influence plasma concentrations of a number of haemostatic and fibrinolytic factors including t-PA, PAI-1 and fibrinogen [de Lange *et al* 2001]. A common intronic polymorphism of the t-PA gene is generated by the insertion of a 311 base pair *Alu*-repeat sequence [Ludwig *et al* 1992]. Although not a consistent finding in all populations [Ridker *et al* 1997a], there is an independent association between the Ins genotype and the future risk of MI: a relative risk of 2.24 in comparison with the Del homozygotes [van der Bom *et al* 1997]. This *Alu*-intronic sequence is now known to be in linkage disequilibrium with three single nucleotide polymorphisms associated with t-PA release within the upstream enhancer region (7,351 C→T), in exon 6 (20,099 T→C) and within intron 10 (27,445 T→A) [Ladenvall *et al* 2000]. The C→T substitution within the enhancer region disrupts a binding site for the Sp1 transcription factor and appears to be functionally important [Ladenvall *et al* 2000; Wolf *et al* 2005] with TT homozygotes having an increased

risk of both MI [Ladenvall *et al* 2002] and lacunar stroke [Jannes *et al* 2004] compared to CC homozygotes.

Genetic differences in t-PA release might therefore alter the fibrinolytic balance predisposing to local thrombus formation and an increased likelihood of adverse atherothrombotic events. Jern and colleagues previously demonstrated a link between t-PA release and both the *Alu*-Ins/Del and -7,351 C/T enhancer polymorphisms in healthy volunteers [Jern *et al* 1999; Ladenvall *et al* 2000]. However the functional importance of these genetic variants in patients with established CHD is unknown. We hypothesised that polymorphisms of the t-PA gene may influence endothelial vasomotor and fibrinolytic function in patients with stable CHD.

4.3 METHODS

4.3.1 SUBJECTS

We recruited patients with CHD confirmed by angiography (defined as $\geq 50\%$ luminal stenosis of at least one major epicardial coronary vessel) or a history of Q-wave MI. All patients had stable anginal symptoms and had not undergone coronary revascularisation within the preceding 3 months. Exclusion criteria were significant cardiac failure, renal impairment, systolic blood pressure <100 or >190 mmHg, impaired fasting glucose (>7 mmol/L) or prior diagnosis of diabetes mellitus. Self-reported smoking status was confirmed using plasma cotinine levels [Feyerabend and Russell 1990]. All studies were undertaken with the approval of the local Research Ethics Committee, the written informed consent of each subject, and in accordance with the Declaration of Helsinki.

4.3.2 DRUGS

Substance P (Clinalfa AG, Switzerland) and sodium nitroprusside (David Bull Laboratories, UK) were administered following dissolution in 0.9% saline. All solutions were freshly prepared on the day of study.

4.3.3 FOREARM STUDY PREPARATION

Subjects were requested to abstain from alcohol for 24 hours and food, caffeine-containing drinks and tobacco for at least 4 hours before each study. Cardio-active medications were withheld on the morning of each study visit. All studies were conducted in a quiet temperature-controlled room maintained at 22-25°C. Patients

rested recumbent, strain gauges and cuffs were applied. Venous cannulae were inserted in each arm and the brachial artery of the non-dominant arm cannulated. Non-invasive blood pressure and heart rate were measured throughout each study.

4.3.4 STUDY DESIGN

Following a 30 minute saline infusion to allow equilibration and recording of resting blood flow, intra-arterial substance P (2, 4 and 8 pmol/min) and sodium nitroprusside (2, 4 and 8 µg/min) were each administered for 6 minutes in a randomised order with a 15 minute saline washout period between each agent. Forearm blood flow was measured at baseline and with each dose of vasodilator; plasma t-PA and PAI-1 antigen concentrations at baseline and with each dose of substance P.

4.3.5 GENOTYPING

Genomic DNA was isolated from whole blood. For the -7,351 C→T (upstream enhancer), 20,099 T→C (exon 6), and 27,445 T→A (intron 10) polymorphisms genotyping was performed by allele discrimination analysis using the 5' nuclease assay using previously published DNA and primer sequences [Ladenvall *et al* 2000; Ladenvall *et al* 2002]. For the *Alu*-intronic polymorphism, the insertion specific sequence was submitted to the Applied Biosystems Assay-by-design service. An insertion specific probe was labelled in its 5' end with FAM and in its 3' end with a NFQ (non fluorescent quencher). Real time quantitative PCR was performed with a ABI PRISM® 7900HT Sequence Detector and *Alu*-Ins PCR products normalised to threshold values against an endogenous control. Each sample was run in triplicate with both the gene of interest and the RNase P control. Genotype for the *Alu*-intronic

polymorphism was calculated from differences in the threshold cycle between the *RNase P* and *Alu-Ins* sequence.

A confirmatory assay of *Alu-Ins/Del* genotypes utilised PCR amplification of the *Ins* fragment with primers designed to flank the region of interest and PCR performed on a DNA engine tetrad from MJ Research (Bio-Rad Laboratories Inc., USA). Amplification of the products produces a 570-bp fragment from chromosomes with the *Ins* allele and a 260-bp fragment from those without (*Del*). All genotyping was performed in triplicate by an individual without information on case status or vascular study data.

4.3.6 STATISTICAL ANALYSIS

Allele frequencies were estimated by gene counting. Chi-square test was used to evaluate deviations of genotype distributions from the Hardy-Weinberg equilibrium. The linkage disequilibrium coefficient D' was calculated as previously described [Devlin and Risch 1995]. Baseline characteristics of the study population are presented as frequencies or mean values and SEM. Differences between groups were compared by Student's *t*-test, Chi-squared test, and, where appropriate, by ANOVA with repeated measures. As C-reactive protein values were not normally distributed they were log transformed prior to analysis. All statistical calculations were undertaken using GraphPad Prism (GraphPad Software, California, USA). Statistical significance was assigned at the 5% level.

4.4 RESULTS

A total of 96 patients were recruited with a cardiovascular risk factor profile in keeping with their diagnosis of CHD and most were prescribed secondary preventative medications.

4.4.1 GENE FREQUENCY

The genotype distributions for the *Alu*-Ins/Del (II 46%, ID 36% and DD 18%), -7,351 C/T enhancer (CC 54%, CT 32% and TT 14%), 20,099 T/C exon 6 (TT 42%, CT 43% and CC 15%) and 27,445 T/A intron 10 (TT 56%, TA 34% and AA 10%) polymorphisms were in keeping with the Hardy-Weinberg equilibrium. There was significant linkage disequilibrium between the four polymorphisms (Table 4.1) with allelic frequencies being similar to published studies of European Caucasian subjects [Jern *et al* 1999; Ladenvall *et al* 2000). There was complete agreement between the two PCR methods in the determination of *Alu*-Ins/Del genotype. Three subjects could be genotyped only for the *Alu*-Ins/Del variant.

Biometric variables were similar between genotypes (Tables 4.2 and 4.3, data on file) although there were significantly fewer smokers in the -7,351 heterozygote group ($p=0.01$, Chi-squared).

Table 4.1 Allele frequencies and pairwise linkage disequilibrium (LD) coefficient between polymorphisms at the tissue plasminogen activator (t-PA) locus; (Chi-squared, degrees of freedom), $p < 0.01$ for all

		Allelic frequency	Alu	-7,351	20,099	27,445
<i>Alu</i>	Ins→Del	0.65/0.35		0.63	0.83	0.60
				(67.4; 4)	(107.8;4)	(76.7; 4)
-7,351	C→T	0.69/0.31			0.85	0.68
					(93.7; 4)	(82.3; 4)
20,099	T→C	0.63/0.37				0.66
						(98.5; 4)
27,445	T→A	0.73/0.27				

4.4.2 HAEMODYNAMICS AND FOREARM BLOOD FLOW

Resting heart rate, blood pressure and non-infused FBF were similar in each group and these did not change significantly during infusion of either vasodilator (Tables 4.2, 4.3 and 4.4; data on file).

Substance P and sodium nitroprusside increased the infused FBF in all subjects ($p < 0.001$, ANOVA). None of the genetic polymorphisms appeared to influence the endothelium-dependent or -independent blood flow responses (Figure 4.1; data on file).

Table 4.2 Clinical characteristics of subjects according to *Alu*-Ins/Del genotype (n=96)

	Genotype			P [†]
	II n=44	ID N=35	DD n=17	
Age, years	59 ± 1	60 ± 1	59 ± 2	0.88
Gender, male/female	33/11	32/3	14/3	0.16
BMI, kg/m ²	29 ± 1	28 ± 1	30 ± 1	0.27
Systolic BP, mmHg	136 ± 3	128 ± 4	132 ± 4	0.23
Diastolic BP, mmHg	79 ± 2	75 ± 2	73 ± 2	0.30
Pulse, bpm	57 ± 1	58 ± 2	57 ± 3	0.96
Current smoker, (%)	30	23	47	0.42
Co-morbidity, (%)				
Previous MI	43	46	24	0.28
Hypertension	50	49	47	0.98
FHx CHD	52	37	29	0.74
Prior hyperlipidaemia	93	94	94	1.00
Extent of coronary disease, (%)*				
1 vessel	41	51	41	0.33
2 vessels	27	20	24	0.86
3 vessels	30	17	35	0.43
Medical Therapy, (%)				
Aspirin	100	100	94	0.10
Statin therapy	91	97	94	0.52
ACE inhibitor, ARB	27	34	29	1.00
Urea, mmol/L	5.4 ± 0.2	5.6 ± 0.3	5.7 ± 0.4	0.70
Creatinine, µmol/L	93.1±2.5	94.6±2.1	91.3±2.6	0.72
Glucose, mmol/L	5.6 ± 0.1	5.7 ± 0.2	6.0 ± 0.2	0.24
Total chol, mmol/L	4.5 ± 0.2	4.4 ± 0.2	4.4 ± 0.2	0.89
LDL chol, mmol/L	2.6 ± 0.1	2.5 ± 0.2	2.2 ± 0.2	0.28
Triglycerides, mmol/L	1.7 ± 0.2	1.7 ± 0.2	2.3 ± 0.3	0.09
CRP, mg/L	1.87±0.18	1.92±0.35	1.90±0.41	0.97

Values are mean ± SEM

[†]P= ANOVA or Chi-squared for difference between genotypes. *Angiographic data unavailable on 5 subjects.

BMI – body mass index; bpm – beats per minute; MI – myocardial infarction; FHx – family history; CHD – coronary heart disease; ACE – angiotensin-converting enzyme, ARB – angiotensin receptor blocker, chol – cholesterol; LDL – low-density lipoprotein; CRP – C-reactive protein.

Table 4.3 Clinical characteristics of subjects according to -7,351 C/T genotype (n=93)

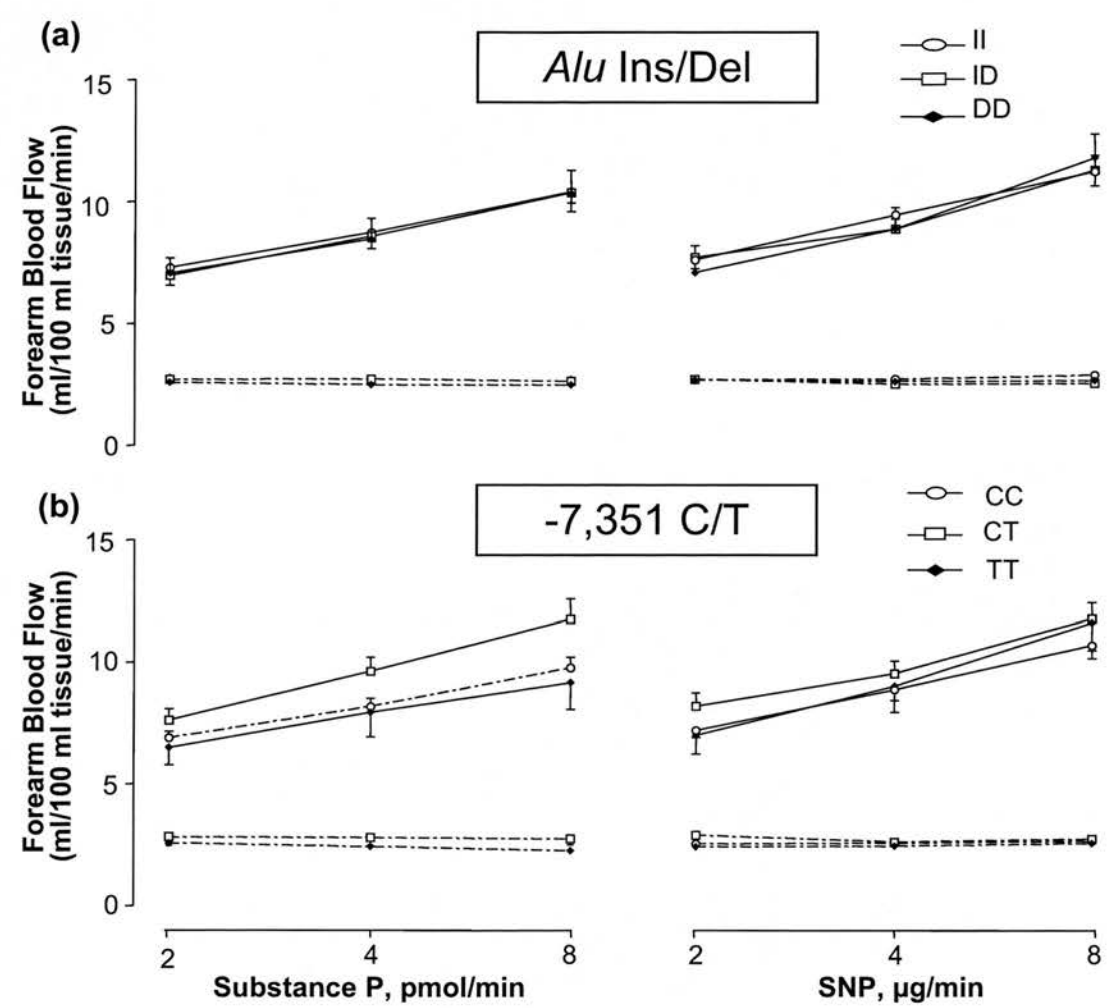
	Genotype			P [†]
	CC n=50	CT n=30	TT n=13	
Age, years	59 ± 1	59 ± 1	61 ± 3	0.82
Gender, male/female	38/12	27/3	11/2	0.28
BMI, kg/m ²	29 ± 1	28 ± 1	29 ± 1	0.47
Systolic BP, mmHg	134 ± 3	131 ± 4	123 ± 6	0.27
Diastolic BP, mmHg	79 ± 2	75 ± 2	70 ± 3	0.07
Pulse, bpm	57 ± 1	57 ± 2	54 ± 3	0.57
Current smoker, (%)	46	13	38	0.01
Co-morbidity, (%)				
Previous MI	44	43	38	0.89
Hypertension	46	40	62	0.43
FHx CHD	32	37	31	0.23
Prior hyperlipidaemia	92	93	92	0.98
Extent of coronary disease, (%)*				
1 vessel	40	50	38	0.64
2 vessels	24	23	23	0.99
3 vessels	30	20	38	0.42
Medical Therapy, (%)				
Aspirin	100	97	100	0.35
Statin therapy	88	100	92	0.14
ACE inhibitor, ARB	28	23	54	0.98
Urea, mmol/L	5.2 ± 0.2	5.7 ± 0.2	6.1 ± 0.5	0.09
Creatinine, µmol/L	93.3 ± 2.2	92.6 ± 2.1	92.3 ± 4.0	0.96
Glucose, mmol/L	5.6 ± 0.1	5.6 ± 0.2	6.0 ± 0.3	0.26
Total chol, mmol/L	4.6 ± 0.2	4.4 ± 0.2	4.2 ± 0.3	0.37
LDL chol, mmol/L	2.7 ± 0.1	2.6 ± 0.1	2.0 ± 0.3	0.08
Triglycerides, mmol/L	1.8 ± 0.2	1.6 ± 0.1	2.4 ± 0.3	0.08
CRP, mg/L	2.02±0.29	1.5±0.3	2.3±0.7	0.49

Values are mean ± SEM

[†]P= ANOVA or Chi-squared for difference between genotypes. *Angiographic data unavailable on 5 subjects.

BMI – body mass index; bpm – beats per minute; MI – myocardial infarction; FHx – family history; CHD – coronary heart disease, ACE – angiotensin converting enzyme; ARB – angiotensin receptor blocker; chol – cholesterol; LDL – low-density lipoprotein; CRP – C-reactive protein.

Figure 4.1 Infused (solid line) and non-infused (dashed line) forearm blood flow (FBF) during intra-brachial substance P (left) and sodium nitroprusside (SNP; right) according to (a) *Alu* Ins/Del and (b) -7,351 C/T, genotypes.
 $p < 0.001$ ANOVA, for infused FBF response to both vasodilators.



4.4.3 PLASMA FIBRINOLYTIC VARIABLES

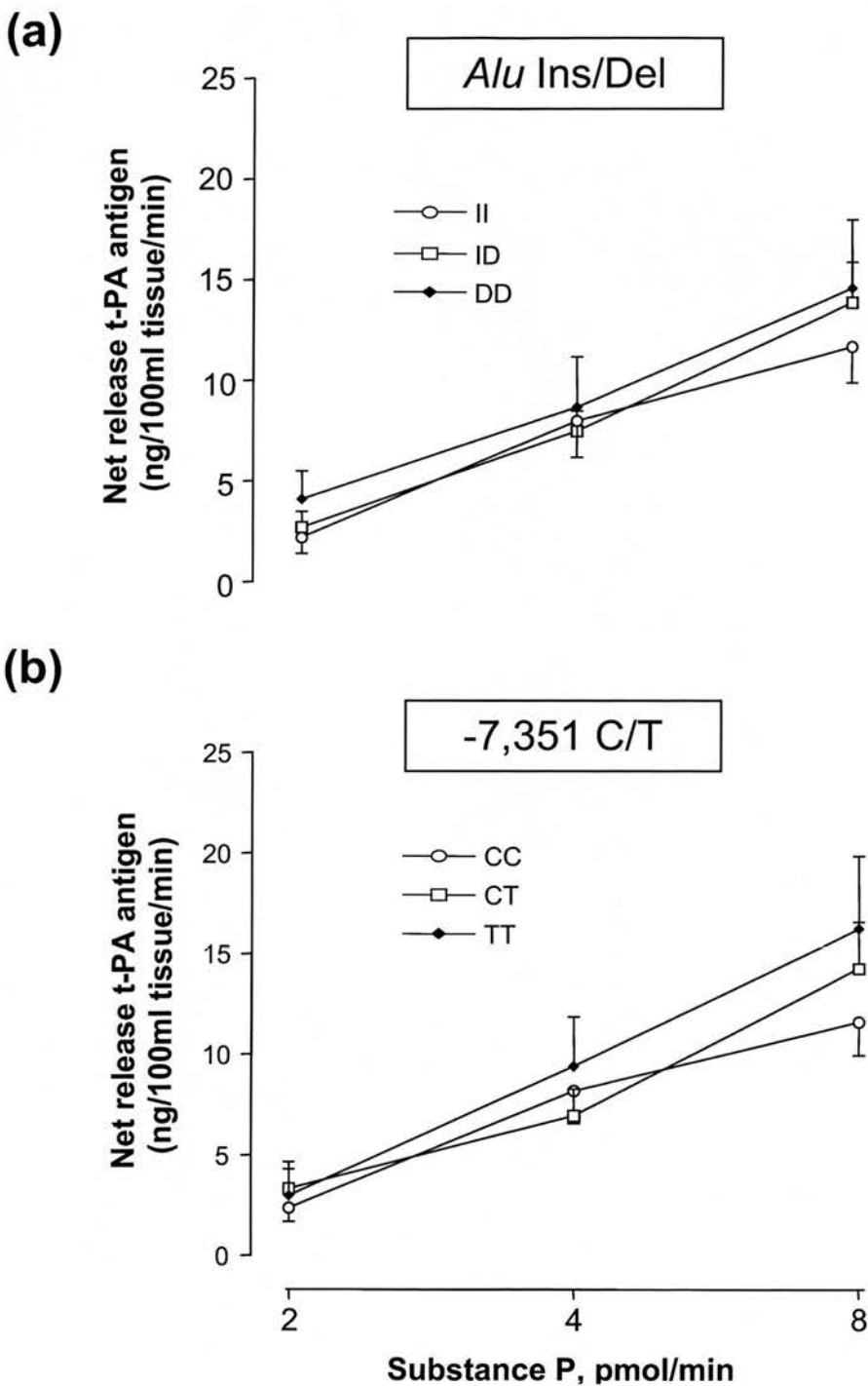
Baseline plasma t-PA and PAI-1 antigen concentrations were similar for each genotype (Table 4.4; data on file). Substance P caused a dose-dependent increase in plasma t-PA antigen within the infused arm in all three groups ($p < 0.01$, ANOVA) whilst plasma PAI-1 antigen concentrations were unchanged (data on file). There were no differences in substance P-induced t-PA release between any of the genetic polymorphisms (Figure 4.2; data on file). Similarly, the area under the curve for net t-PA release over the 30 minute period of substance P infusion was similar between genotypes (data on file).

Table 4.4 Effect of *Alu*-Ins/Del and -7,351 C/T genotypes on baseline forearm blood flow, tissue plasminogen activator (t-PA) and plasminogen activator inhibitor type 1 (PAI-1) antigen concentrations; $p = ns$ between genotypes

	<i>Alu</i> -Ins/Del			-7,351 C/T		
Forearm blood flow (ml/100ml tissue/min)	II	ID	DD	CC	CT	TT
Infused arm	2.7±0.1	2.8±0.2	2.7±0.2	2.6±0.1	2.8±0.2	2.6±0.3
Non-infused arm	2.7±0.2	2.7±0.2	2.5±0.2	2.6±0.1	2.9±0.2	2.4±0.3
t-PA Antigen (ng/mL)						
Infused arm	9.1±0.5	10.7±0.8	9.0±0.7	9.4±0.5	9.8±0.8	9.3±1.1
Non-infused arm	9.3±0.5	10.9±0.8	8.8±0.7	9.6±0.5	9.9±0.8	9.3±1.1
PAI-1 Antigen (ng/mL)						
Infused arm	43.3±4.2	43.6±3.7	44.1±6.6	44.5±3.5	44.3±4.8	39.8±7.4
Non-infused arm	46.0±4.2	44.0±3.6	43.7±6.5	47.7±3.6	42.4±4.6	40.0±7.2

Values are mean ± SEM

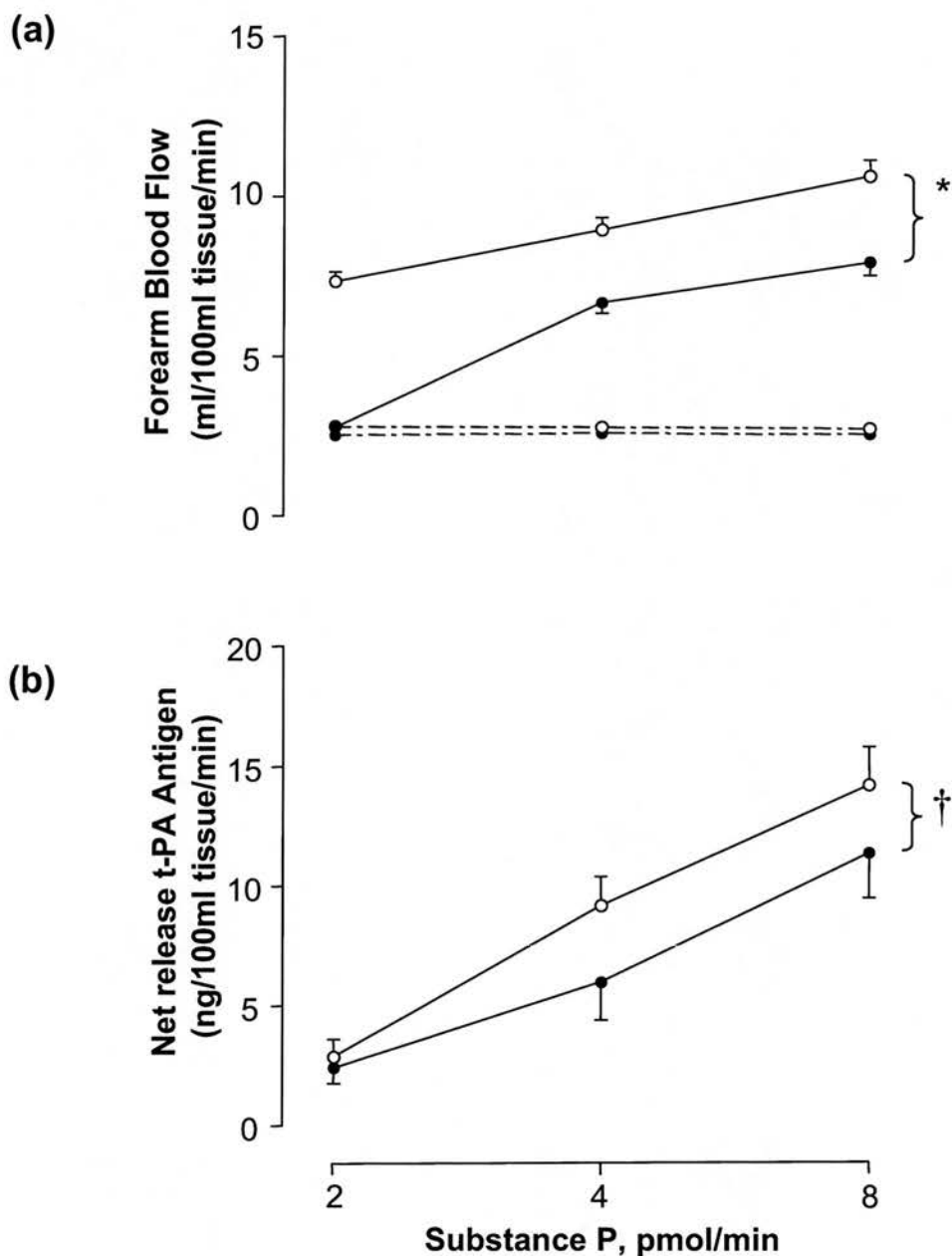
Figure 4.2 Estimated net release of t-PA antigen induced by intra-brachial substance P (2, 4 and 8 pmol/min) according to (a) *Alu* Ins/Del, and (b) -7,351 C/T, genotypes. $p<0.001$, ANOVA, for dose response.



Smoking status was confirmed by plasma cotinine concentration (median 307.8 *versus* 0.6 ng/mL in smokers and non-smokers respectively). Subgroup analysis revealed an interaction between smoking status and vascular function. Consistent with our previous findings, cigarette smokers had impaired FBF responses ($p < 0.001$, ANOVA) and net t-PA release ($p = 0.05$, ANOVA; Figure 4.3) compared to non-smokers. In light of the uneven distribution of smokers across -7,351 genotypes and the interaction between smoking and t-PA release, we performed a post-hoc analysis of t-PA release according to smoking status. However we found no difference in the area under the curve for net t-PA release between CC, CT and TT -7,351 genotypes in either smokers (111 [48-174]; 167 [94-240]; 193 [5-382]; $p = 0.41$ ANOVA) or non-smokers (187 [114-260]; 160 [100-220]; 188 [50-325]; $p = 0.83$) respectively (Mean [95% confidence intervals]).

Figure 4.3 Infused (solid line) and non-infused (dashed line) forearm blood flow (a), and net t-PA release (b), in response to intra-brachial substance P in smokers (●) and non-smokers (○).

* $p < 0.001$, 2-way ANOVA, infused FBF response in smokers *versus* non-smokers, † $p = 0.05$, 2-way ANOVA, net t-PA release in smokers *versus* non-smokers.



4.5 DISCUSSION

This is the first study to assess directly the role of t-PA genetic polymorphisms in determining acute t-PA release in patients with CHD. Using a validated and reproducible technique [Oliver *et al* 2005], we have found no difference in the basal concentration, or net release of, plasma t-PA antigen between genotypes. These important findings suggest that, in patients with established coronary atherosclerosis, variation at the t-PA gene locus does not substantially influence acute endogenous fibrinolytic capacity.

Tissue plasminogen activator release from endothelial cells is the major arbiter of local intravascular fibrinolysis [Kooistra *et al* 1994] and will influence vessel patency following acute coronary thrombosis [Rosenberg and Aird 1999]. Inter-individual differences in t-PA release may contribute to a pro-thrombotic phenotype and determine the sequelae of acute MI. Indeed, clustering of individuals with low rates of t-PA release has been reported in families with a history of thrombosis [Petaja *et al* 1991]. Moreover, genetic murine models of t-PA deficiency show a reduced thrombolytic potential and an increased thrombotic tendency [Carmeliet *et al* 1994]. We therefore wanted to explore the potential genetic influences on t-PA release in at risk patients with CHD.

The tissue plasminogen activator -7,351 C→T enhancer site polymorphism has been associated with an increased risk of first MI [Ladenvall *et al* 2002] and lacunar stroke [Jannes *et al* 2004] although the data on non-lacunar stroke is conflicting

[Jannes *et al* 2004; Jood *et al* 2005]. The strong linkage between the -7,351 C/T enhancer and the *Alu*-repeat polymorphisms may explain the association between the (non-coding) *Alu*- Ins/Del polymorphism and cardiovascular events [van der Bom *et al* 1997; Ladenvall *et al* 2000]. In healthy volunteers (n=51), both the *Alu* Ins/Del and -7,351 C→T enhancer polymorphisms were associated with significant differences in basal t-PA release with Ins and C homozygotes having around 2-fold greater release rates than Del and T homozygotes respectively [Jern *et al* 1999; Ladenvall *et al* 2000]. Of the genotypes studied here, the 7,351 C→T substitution is thought to result in a functional change occurring within an Sp1 transcription factor binding site which is negatively regulated by binding of the thymidine allele [Ladenvall *et al* 2000]. However despite this difference in basal t-PA release rate, acute t-PA release was similar between all genotypes. Now, in a larger cohort of subjects (n=96) with established CHD, we have shown that there is no significant relationship between basal plasma t-PA concentrations or acute t-PA release, and these genetic polymorphisms. Furthermore, endothelium-dependent vasodilatation, a complementary measure of endothelial function, was similarly unaffected by variation within the t-PA gene locus.

Regulatory control of t-PA release involves distinct pathways from those controlling vessel calibre and indeed t-PA release can be induced without changes in FBF [Chia *et al* 2003b]. In view of previous work in healthy volunteers [Jern *et al* 1999; Ladenvall *et al* 2000], we chose to assess whether a number of t-PA genetic polymorphisms were associated with changes in vascular function in patients with CHD. If t-PA genotype was important in regulating endogenous t-PA release, certain

alleles could favour the continued presence of thrombus following atheromatous plaque rupture. Organisation of residual thrombus is known to stimulate further plaque growth and expansion [Mann and Davies 1999] disrupting endothelial integrity which is critical to the regulation of vascular tone and coagulation. Impaired endothelium-dependent vasodilatation is considered a surrogate of endothelial dysfunction and has been widely studied as a bio-marker of atherosclerosis. We therefore hypothesised that t-PA genetic polymorphisms might also be associated with changes in endothelium-dependent vasodilatation through pathways linking coagulation, atherogenesis, and endothelial dysfunction. However we found no evidence to support the contention that t-PA polymorphisms influence endothelial function in patients with CHD. In contrast, cigarette smoking and CHD are associated with endothelial dysfunction as demonstrated by impaired endothelium-dependent vasodilatation [Celermajer *et al* 1993] and endogenous t-PA release [Newby *et al* 1999; Newby *et al* 2001]. Our study confirms these previous findings in cigarette smokers in a large cohort of patients with established CHD maintained on secondary preventative medications. These consistent findings would appear to reaffirm the validity of our approach and suggest that environmental rather than genetic factors have a more dominant influence on acute endogenous t-PA release.

There are a number of differences between the current study and previous reports demonstrating an interaction between acute t-PA release and polymorphisms within the t-PA gene. Our population was comprised of subjects with established CHD recruited within the United Kingdom. In contrast, Jern *et al* [Jern *et al* 1999; Ladenvall *et al* 2000] studied healthy Scandinavian volunteers free of symptomatic

atherosclerosis. A recent meta-analysis of studies on genetic polymorphisms and CHD risk demonstrated heterogeneity according to the population studied [Casas *et al* 2004]. Indeed, the *Alu*-repeat polymorphism was associated with an increased incidence of acute MI in Dutch subjects [van der Bom *et al* 1997] but not in studies from North America [Ridker *et al* 1997a] or the United Kingdom [Steeds *et al* 1998]. Our subjects were all of European Caucasian origin, recruited from within the same geographical area and had similar risk factor profiles. It seems unlikely that biological variability or modulation from environmental factors obscured true differences in t-PA release between genotypes. Although our findings may not be applicable to other racial groups, Rosenbaum and colleagues [Rosenbaum *et al* 2002] failed to demonstrate differences in acute endogenous t-PA release between healthy black and white Americans.

4.5.1 STUDY LIMITATIONS

Although endothelium-dependent blood flow responses are similar in the forearm and coronary circulations [Anderson *et al* 1995], we cannot be sure that these polymorphisms of the t-PA gene do not influence fibrinolytic responses within the coronary circulation. Indeed, there are limited data to suggest that, although not apparent in the forearm circulation, angiotensin-converting enzyme gene Ins/Del polymorphism can influence acute t-PA release in the coronary circulation [Ohira *et al* 2004] .

Jern and colleagues previously demonstrated a difference in basal, though not stimulated, t-PA release according to t-PA genotype [Jern *et al* 1999; Ladenvall *et al*

2000]. Whilst we cannot determine basal t-PA release using the venovenous technique described, we have found no relationship between t-PA genotype and stimulated t-PA release. Calculation of net t-PA release provides an accurate assessment of stimulated t-PA release with good reproducibility [Newby *et al* 2002], and basal release contributes only a small proportion of the overall venous plasma t-PA concentration. In this study we did not find any difference in acute t-PA release in response to substance P between t-PA genotypes. However, our study protocol cannot exclude the possibility that these polymorphisms might alter protein transcription and endothelial storage, or the very early (<6 minutes) release of, t-PA in response to substance P infusion [Ridderstrale *et al* 2006].

In our cohort, there was an uneven distribution of smokers between t-PA -7,351 CC, CT and TT groups. Although we found no difference in net t-PA release between these three groups when analysed according to smoking status, we accept that the relationship between cigarette smoking and impaired t-PA release may have obscured an association between t-PA genotype and fibrinolytic function with the post-hoc analysis limited by the number of smokers and non-smokers within each group.

In summary, whilst we have confirmed previous findings showing that cigarette smokers have impaired endothelial vasomotor and fibrinolytic function, we have found no major effect of variation within the t-PA gene locus on either plasma t-PA concentrations or acute t-PA release in subjects with stable CHD.

CHAPTER 5

VASCULAR AND FIBRINOLYTIC EFFECTS OF INTRA-ARTERIAL TUMOUR NECROSIS FACTOR- α IN PATIENTS WITH CORONARY HEART DISEASE

Robinson SD, Dawson P, Ludlam CA, Boon NA, Newby DE.
Vascular and fibrinolytic effects of intra-arterial tumour necrosis
factor-alpha in patients with coronary heart disease.
Clin Sci (Lond) 2006;**110** (3):353-60.

5.1 SUMMARY

Elevated plasma t-PA and serum CRP concentrations are associated with an adverse cardiovascular risk. We investigated whether acute local inflammation causes vascular dysfunction, and influences t-PA release in patients with stable CHD. Serum CRP, plasma t-PA and PAI-1 concentrations were determined in 95 patients with stable CHD. A representative sub-population of 12 male patients received an intra-brachial infusion of TNF- α and saline placebo using a randomised double-blind crossover study design. Forearm blood flow, and plasma fibrinolytic and inflammatory variables were measured. Serum CRP concentrations correlated with plasma t-PA concentrations ($r=0.37$, $p<0.001$) and t-PA/PAI-1 ratio ($r= -0.21$, $p<0.05$). Intra-arterial TNF- α caused a rise in t-PA concentrations ($p<0.001$) without affecting blood flow or PAI-1 concentrations. Tumour necrosis factor- α pre-treatment impaired acetylcholine and nitroprusside-induced vasodilatation ($p<0.001$ for both) whilst doubling bradykinin-induced t-PA release ($p=0.006$). In patients with stable CHD, plasma fibrinolytic factors correlate with a systemic inflammatory marker, and local vascular inflammation directly impairs vasomotor function whilst enhancing endothelial t-PA release. We suggest that the adverse prognosis associated with elevated plasma t-PA concentrations relates to the underlying causative association with vascular inflammation and injury.

5.2 INTRODUCTION

In epidemiological studies of patients with CHD [Thompson *et al* 1995], and in prospective studies in healthy populations [Ridker *et al* 1993], higher plasma concentrations of the pro-fibrinolytic factor t-PA positively and independently predict future cardiovascular events. It would be anticipated that high t-PA concentrations would protect against subsequent cardiovascular events rather than the reverse. This paradoxical association is, in part, explained by the concomitant elevation of PAI-1 which complexes with, and inactivates, t-PA. However, the precise stimulus for this increased t-PA release remains unclear.

Areas of endothelial denudation and thrombus deposition are a common finding on the surface of atheromatous plaques and are usually subclinical. Through t-PA release, endogenous fibrinolysis is usually able to prevent thrombus propagation although organisation of the residual thrombus may lead to plaque growth and expansion [Mann and Davies 1999]. The adverse prognosis conferred by elevated plasma t-PA antigen concentrations may therefore reflect the extent of occult atheroma and subclinical plaque rupture stimulating t-PA release.

Markers of systemic inflammation, such as CRP and TNF- α , are elevated in patients with cardiovascular disease [Haverkate *et al* 1997; Ridker *et al* 2000a]. Indeed, serum CRP concentrations predict the development of cardiovascular disease independent of other risk factors. Previous studies have indicated a direct relationship between serum CRP and plasma t-PA concentrations [Haverkate *et al*

1995; Lowe *et al* 2004]. This raises the question of whether vascular inflammation is causally related to the elevation in plasma t-PA concentrations or whether CRP and t-PA are independently increased by a common factor related to the atherosclerotic process itself, such as acute plaque rupture.

Abnormalities of endothelial function have been demonstrated in patients with atherosclerosis [Ludmer *et al* 1986] and vascular inflammation [Hingorani *et al* 2000b; Chia *et al* 2003a; 2003b]. In patients with CHD, restoration of endothelium-dependent vasomotor function occurs when there is normalisation of CRP concentrations [Fichtlscherer *et al* 2000] whereas ongoing chronic inflammation is associated with an impaired fibrinolytic response to venous occlusion [Speidl *et al* 2005]. As the endothelium is the major source of plasma t-PA, abnormalities of endothelial function may therefore mediate the potential inflammation-induced elevations in plasma t-PA concentrations.

It therefore remains unclear whether elevated t-PA concentrations are implicated in the mechanisms contributing to, or arise as a consequence of, atherothrombotic events. The aims of the present study were, in patients with stable CHD, to confirm the previous association between plasma CRP and t-PA concentrations, and to determine the effect of acute local vascular inflammation provoked by direct intra-arterial infusion of TNF- α on vasomotor function and endothelial t-PA release.

5.3 METHODS

5.3.1 PATIENTS

We recruited patients with CHD confirmed by angiography (defined as $\geq 50\%$ luminal stenosis of at least one major epicardial coronary vessel) or a previous history of Q-wave MI. All patients had stable anginal symptoms and had not undergone coronary revascularisation within the preceding 3 months. Exclusion criteria were significant cardiac failure, renal impairment, systolic blood pressure < 100 or > 190 mmHg, diabetes mellitus, history or clinical features of recent infective illness and immunosuppressive or non-steroidal anti-inflammatory medications (excluding aspirin 75 mg/d). All studies were undertaken with the approval of the local Research Ethics Committee, the written informed consent of each subject, and in accordance with the Declaration of Helsinki.

5.3.2 DRUGS

Tumour necrosis factor- α (Knoll Pharmaceuticals, Germany), bradykinin (Clinalfa, AG, Switzerland), acetylcholine (Novartis UK Ltd, UK), and sodium nitroprusside (David Bull Laboratories, UK) were administered following dissolution in 0.9% saline.

5.3.3 STUDY DESIGN

All subjects abstained from alcohol for 24 hours and from food, tobacco and caffeine-containing drinks for at least 4 hours before each study visit. A venous blood sample was taken from all patients for estimation of serum CRP, and plasma

t-PA and PAI-1 antigen concentrations. Twelve representative male patients were then recruited into a randomised double-blind placebo-controlled crossover study comparing the effect of direct intra-brachial infusion of TNF- α and saline placebo on FBF and endogenous fibrinolytic function.

5.3.4 FOREARM STUDY PROTOCOL

All studies were carried out at 09:00 hours in a quiet temperature-controlled room maintained at 22-25°C. Patients rested recumbent, strain gauges and cuffs were applied. Venous cannulae were inserted and the brachial artery of the non-dominant arm cannulated with a 27-G needle. Blood pressure and heart rate were measured using a semi-automated non-invasive sphygmomanometer.

Twelve subjects attended on two occasions at least 2 weeks apart and received an intra-arterial infusion of either TNF- α (80 ng/min) or saline placebo over 60 minutes followed on each occasion by a further 60 minutes of saline infusion. Thereafter, intra-arterial bradykinin (100, 300, 1000 pmol/min), acetylcholine (5, 10, 20 μ g/min) and sodium nitroprusside (2, 4, 8 μ g/min) were administered at 1 mL/min with a 15 minute saline washout period between each agent. The dose of TNF- α was chosen to achieve local cytokine concentrations comparable to healthy volunteer studies [Chia *et al* 2003b], and those seen in cardiovascular disease [Biasucci *et al* 1996].

Venous blood samples for t-PA antigen and activity and PAI-1 antigen were obtained at baseline, after 60 minutes of TNF- α /placebo infusion, following the 60 minute infusion of saline, before and during each dose of bradykinin and 15 minutes after

the end of bradykinin infusion. Plasma cytokines, *hs*-CRP and prothrombin F1+2 were assessed prior to, and following the TNF- α /placebo and saline infusions, and at the end of each study.

5.3.5 STATISTICAL ANALYSIS

As basal t-PA concentrations were altered by pre-treatment with TNF- α , net release of t-PA during bradykinin infusion was calculated by subtracting the mean t-PA release before, and 15 minutes after cessation of bradykinin infusion [Chia *et al* 2003b]. The area under the curve [Matthews *et al* 1990] was calculated for the estimated net release of t-PA in response to bradykinin. Data were examined, where appropriate, by ANOVA with repeated measures followed by post-hoc *t*-tests adjusted with a Bonferroni correction for multiple comparisons. Spearman's correlation was used to compare CRP and plasma levels of fibrinolytic factors. As serum CRP concentrations have a skewed distribution, they were logarithmically transformed. All statistical calculations were undertaken using GraphPad Prism (GraphPad Software, USA). Results are expressed as mean \pm SEM unless otherwise stated; statistical significance was assigned at the 5% level.

5.4 RESULTS

Patients had a typical cardiovascular risk factor profile in keeping with their diagnosis of CHD and most were prescribed secondary preventative medications (Tables 5.1 and 5.2). The majority of the subjects were male, two-thirds had previously undergone coronary revascularisation and around one-third were habitual smokers.

Table 5.1 Baseline characteristics of the 95 subjects with stable coronary heart disease

Age, years	59 ± 7
Body mass index, kg/m²	29 ± 5
Male gender	79 (83)
Previous myocardial infarction	39 (41)
Extent of coronary artery disease*	
1 vessel	42 (44)
2 vessels	24 (25)
3 vessels	28 (29)
Previous coronary revascularisation	64 (67)
Co-morbidity	
Hypertension	46 (48)
Previous hyperlipidaemia	89 (94)
Family history of premature CHD	32 (34)
Smoker / Ex-smoker / Non-smoker	30 / 27 / 38 (32 / (28) / (40))
Medical Therapy	
Aspirin	95 (100)
Anti-anginal	82 (86)
Statin	90 (95)
ACE inhibitor, ARB	30 (32)
Serum urea, mmol/L	5.6 ± 1.3
Serum creatinine, µmol/L	93 ± 13
Plasma glucose, mmol/L	5.7 ± 0.9
Total cholesterol, mmol/L	4.5 ± 0.9
LDL cholesterol, mmol/L	2.5 ± 0.7
Triglycerides, mmol/L	1.8 ± 0.9

CHD – coronary heart disease; ACE – angiotensin converting enzyme; ARB – angiotensin II receptor blocker; LDL – low-density lipoprotein. *Angiographic data unavailable on 1 subject. All data are mean ± SD or n (%).

Table 5.2 Baseline characteristics of the 12 patients receiving TNF- α and saline placebo

Age, years	60 \pm 2
Body mass index, kg/m²	24 \pm 1
Male gender	12 (100)
Previous myocardial infarction	5 (42)
Extent of coronary disease*	
1 vessel	5 (42)
2 vessels	4 (33)
3 vessels	2 (17)
Previous coronary revascularisation	9 (75)
Co-morbidity,	
Hypertension	3 (25)
Family history of premature CHD	1 (8)
Previous hyperlipidaemia	12 (100)
Smoker / Ex-smoker / Non-smoker	2 / 7 / 3
	(17)/(58)/(25)
Medical Therapy	
Aspirin	12 (100)
Anti-anginal	11 (92)
Statin	12 (100)
ACE inhibitor	1 (8)
Serum urea, mmol/L	5.9 \pm 0.3
Serum creatinine, μ mol/L	93 \pm 2
Plasma glucose, mmol/L	5.2 \pm 0.1
Total cholesterol, mmol/L,	3.9 \pm 0.2
LDL cholesterol, mmol/L	2.1 \pm 0.2
Triglycerides, mmol/L	1.3 \pm 0.2
Placebo visit	
Heart rate, /min	56 \pm 2
Systolic blood pressure, mmHg	145 \pm 6
Diastolic blood pressure, mmHg	83 \pm 4
Forearm blood flow, mL/100 mL/min	
Infused arm	2.1 \pm 0.2
Non-infused arm	2.0 \pm 0.2
TNF-α visit	
Heart rate, /min	56 \pm 3
Systolic blood pressure, mmHg	142 \pm 4
Diastolic blood pressure, mmHg	81 \pm 2
Forearm blood flow, mL/100 mL/min	
Infused arm	2.0 \pm 0.2
Non-infused arm	2.1 \pm 0.2

CHD – coronary heart disease; ACE – angiotensin-converting enzyme; LDL – low-density lipoprotein; TNF- α - tumour necrosis factor- α . *Angiographic data unavailable on 1 subject. Values are mean \pm SEM or n (%).

5.4.1 PLASMA FIBRINOLYTIC FACTORS AND CORRELATION WITH CRP

In the cohort of 95 patients with stable CHD, serum *hs*-CRP concentrations correlated with plasma t-PA antigen concentrations ($r=0.37$, $p<0.001$), plasma PAI-1 antigen concentrations ($r=0.28$, $p=0.006$), and inversely with the ratio of t-PA/PAI-1 antigen ($r= -0.21$, $p<0.05$). As anticipated, plasma t-PA antigen concentrations correlated with plasma PAI-1 antigen concentrations ($r=0.49$, $p<0.001$).

5.4.2 EFFECT OF ACUTE INFLAMMATION ON VASCULAR AND FIBRINOLYTIC FUNCTION

Those subjects who received TNF- α and saline placebo had similar baseline characteristics to the main cohort; all were receiving statin and aspirin therapy. There were no differences in resting arterial pressure, heart rate or FBF between the two study visits (Table 5.2).

There was no change in heart rate or blood pressure during infusion of either TNF- α or placebo. Haematocrit, temperature, white cell count and *hs*-CRP were similar on both study visits ($p=ns$; data on file). The study protocol was well tolerated with no major adverse effects.

5.4.3 PLASMA CYTOKINE CONCENTRATIONS

Intra-arterial infusion of TNF- α increased plasma TNF- α concentrations from 1.4 ± 0.2 to 164.5 ± 26.8 pg/mL in the infused arm and from 1.3 ± 0.1 to 33.3 ± 5.6 pg/mL in the non-infused arm ($p<0.001$; Figure 5.1). One hour after TNF- α infusion, plasma IL-6 concentrations increased from 4.1 ± 1.9 to 6.4 ± 2.3 pg/mL in the

infused arm ($p < 0.001$, ANOVA) but were unchanged in the non-infused arm (4.0 ± 1.9 to 3.7 ± 1.3 pg/mL). Placebo infusion had no effect on plasma IL-6 concentrations in the infused arm (3.9 ± 1.7 to 4.3 ± 1.3 pg/mL, $p = \text{ns}$). Serum CRP concentrations were unchanged following the 60 minute infusion of TNF- α and saline placebo.

5.4.4. VASOMOTOR AND FIBRINOLYTIC RESPONSES

There was no significant change in resting FBF in the 2 hours after the start of either TNF- α or placebo infusion. After 60 minutes of TNF- α , plasma t-PA antigen and activity concentrations in the infused arm had increased from 9.4 ± 1.1 to 11.3 ± 1.2 ng/mL and 0.3 ± 0.1 to 2.1 ± 0.6 IU/mL respectively ($p < 0.001$) and these remained elevated in the 2 hours following discontinuation of the TNF- α infusion (Figure 5.2). There was no change in plasma PAI-1 antigen concentrations in the infused arm, and no change in either plasma t-PA or PAI-1 concentrations in the non-infused arm during the study ($p = \text{ns}$; data on file). Prothrombin F1+2 concentrations in the infused arm were unaltered following either saline placebo (0.9 ± 0.1 to 0.9 ± 0.1 ng/mL; $p = \text{ns}$) or TNF- α infusion (0.9 ± 0.1 to 1.0 ± 0.1 ng/mL; $p = \text{ns}$).

Figure 5.1 Plasma TNF- α concentrations in the infused (solid line) and non-infused arm (dashed line) after 60 minutes of intra-arterial TNF- α (●) and saline placebo (○). $p < 0.001$, ANOVA, TNF- α dose response in the infused arm; TNF- α (●) *versus* saline placebo (○) for infused arm.
TNF- α - tumour necrosis factor- α .

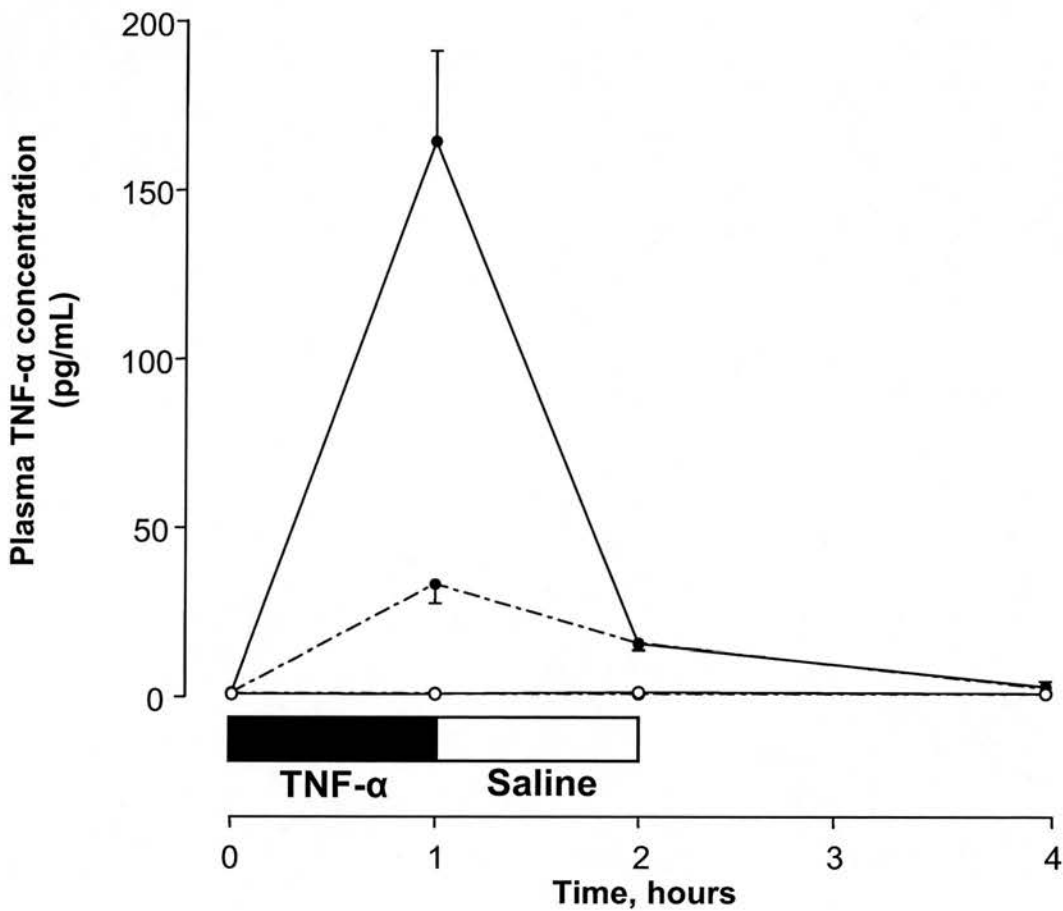
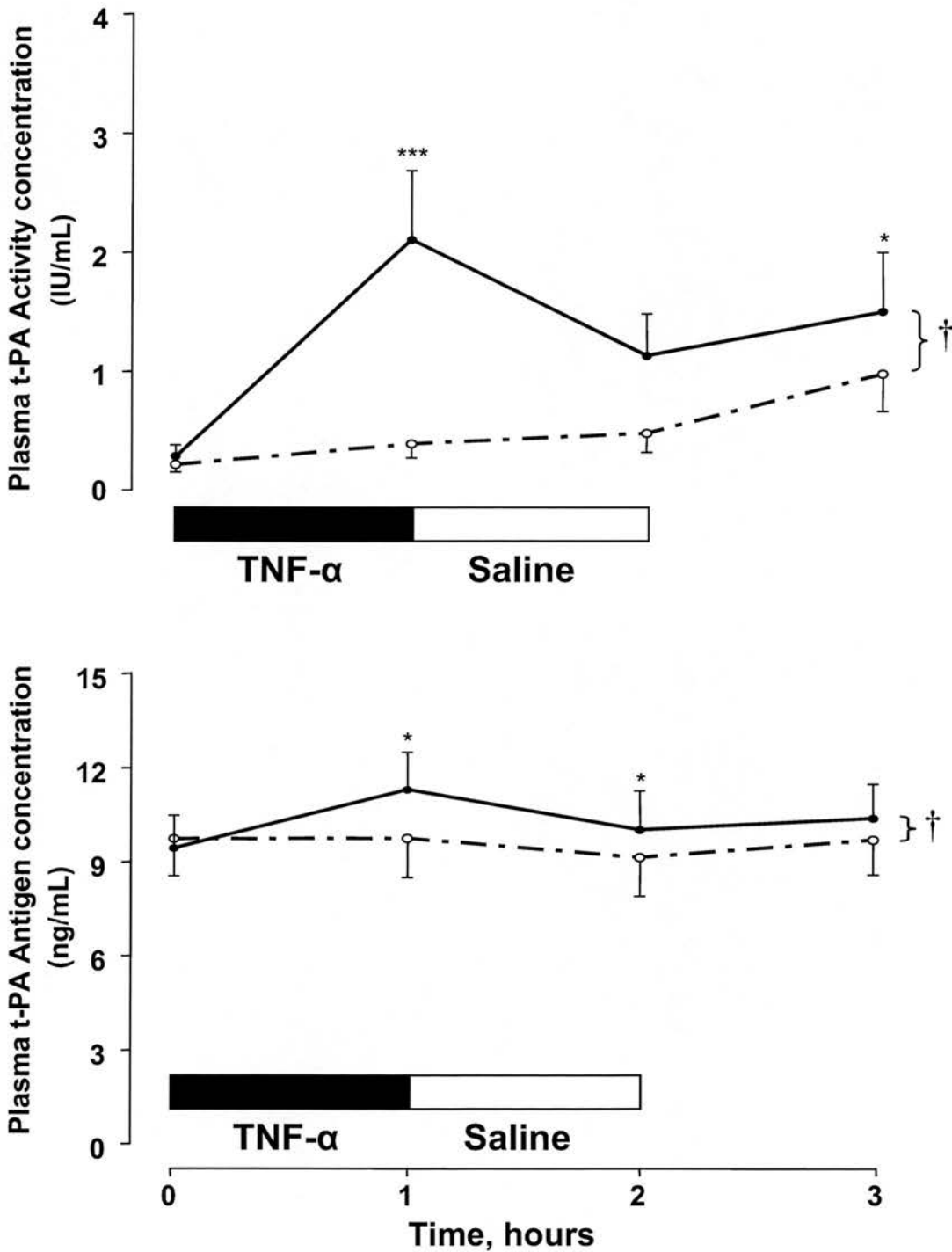


Figure 5.2 Plasma t-PA antigen (bottom), and activity (top), concentrations in the infused (●) and non-infused arms (○) arms after 60 minutes infusion of intra-arterial TNF- α . †p<0.001, ANOVA, infused (●) *versus* non-infused arm (○). *p<0.05 and ***p<0.001, post-hoc *t*-test for treatment effect.
TNF- α - tumour necrosis factor- α .



There was a dose-dependent increase in FBF during bradykinin, acetylcholine and sodium nitroprusside infusion ($p \leq 0.01$, ANOVA). Compared to saline placebo, TNF- α pre-treatment impaired acetylcholine and nitroprusside-induced vasodilatation ($p < 0.001$ for both) but did not alter the response to bradykinin (Figure 5.3).

Plasma t-PA concentrations increased in a dose-dependent manner during bradykinin infusion on both study visits ($p < 0.001$). Pre-treatment with TNF- α augmented the bradykinin-induced rise in plasma t-PA antigen and activity concentrations ($p < 0.001$ for both; Figure 5.4) and significantly increased estimated net release of t-PA antigen and activity (63.7 ± 14.8 versus 120.6 ± 26.1 ng/100 mL tissue/min and 54.8 ± 14.8 versus 98.8 ± 21.0 IU/100 mL tissue/min at peak dose; $p < 0.05$ for both). Over the 30 minute period of bradykinin infusion, TNF- α increased the area under the curve for net t-PA antigen and activity release by 120% and 188% respectively ($p = 0.006$).

Subgroup analysis showed a significant impairment of endothelium-dependent vasodilatation as well as bradykinin-induced t-PA response in cigarette smokers. Qualitatively the effect of intra-arterial TNF- α on the blood flow and fibrinolytic responses were similar in both smokers and non-smokers.

Figure 5.3 Infused (solid line) and non-infused (dashed line) forearm blood flow during incremental doses of acetylcholine (left), sodium nitroprusside (SNP; middle) and bradykinin (right) following pre-treatment with TNF- α (●) or saline placebo (○). $p \leq 0.01$, ANOVA for all infused arm responses. $\dagger p < 0.001$, ANOVA (●) *versus* (○).
 * $p < 0.05$, post-hoc *t*-test for treatment effect; TNF- α - tumour necrosis factor- α .

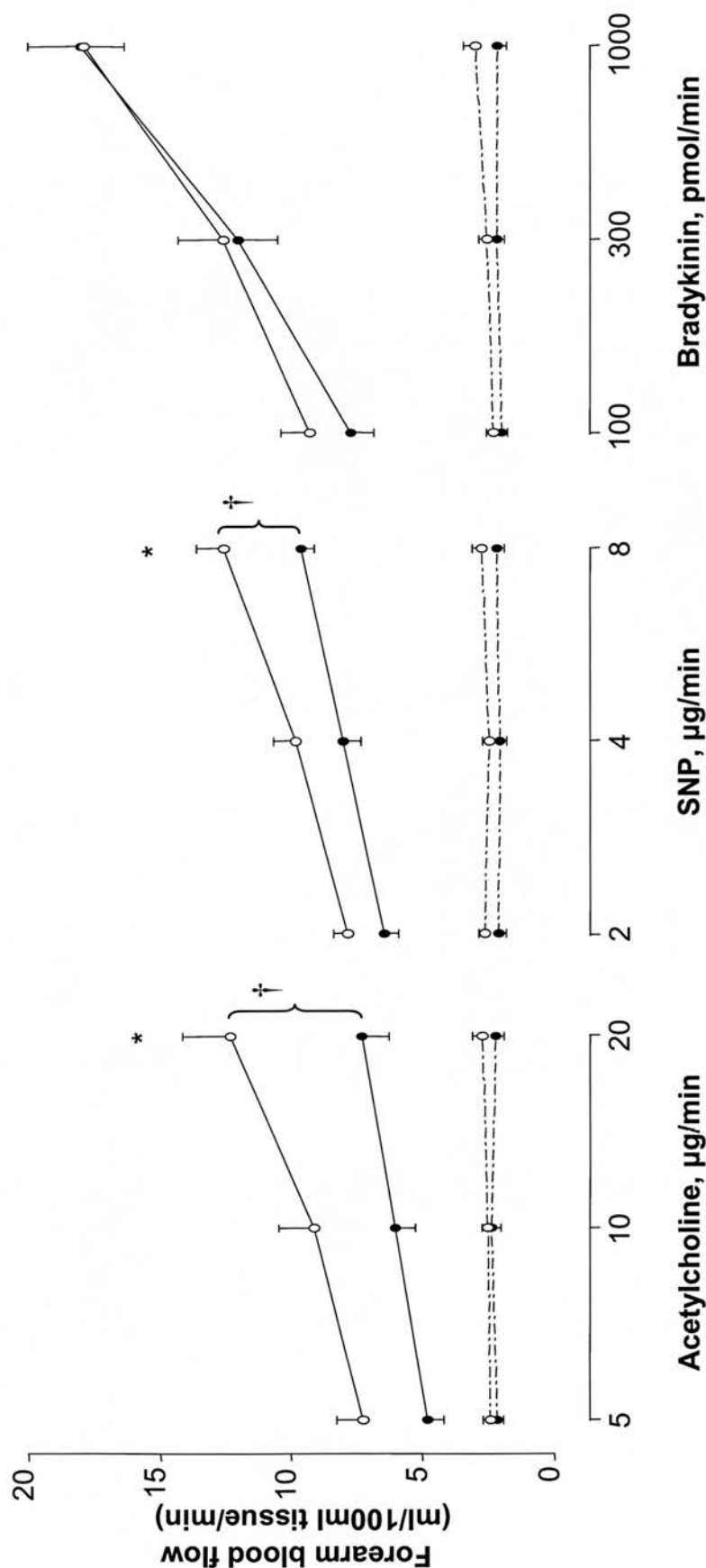
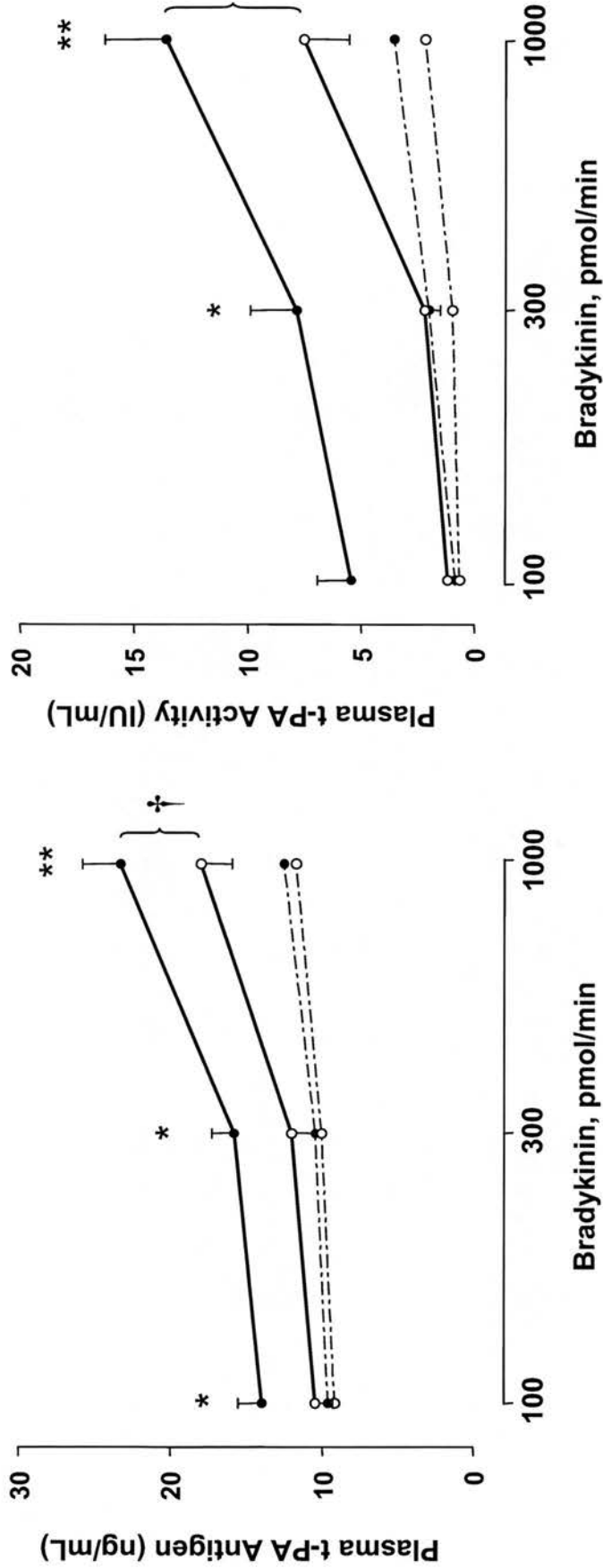


Figure 5.4 Plasma concentrations of t-PA antigen (left), and t-PA activity (right), during bradykinin infusion following pre-treatment with TNF- α (●) or saline placebo (○). †p<0.001, ANOVA (●) versus (○). *p<0.05 and **p<0.01, post-hoc *t*-test for treatment effect.



5.5 DISCUSSION

We have confirmed the direct association between plasma t-PA and serum CRP concentrations in patients with stable CHD. For the first time, we have extended this observation using an acute local vascular inflammatory model and demonstrated that direct intra-arterial infusion of TNF- α causes a slow onset and sustained increase in basal t-PA release. This arterial inflammation was also associated with increased stimulated t-PA release in the presence of impaired vasomotor function. Our findings are consistent with the suggestion that t-PA is released during vascular inflammation and endothelial injury and this may, in part, explain the adverse prognosis associated with increased plasma t-PA concentrations.

The link between markers of inflammation and plasma t-PA concentrations suggests that vascular inflammation and injury may be responsible for endothelial t-PA release. However, the association may arise from common aetiological factors and does not establish a causal relationship. A recent meta-analysis has suggested that increases in plasma t-PA concentrations largely reflect the presence of concomitant cardiovascular risk factors [Lowe *et al* 2004]. We therefore sought to induce vascular inflammation in a representative sample of our study population. Inducing systemic inflammation will have many biological actions and could be confounded by indirect or extra-vascular effects. This may explain some of the differences between our current results and those of previous work in healthy volunteers using *s typhi* vaccination [Hingorani *et al* 2000b; Chia *et al* 2003a]. We chose to employ an acute local inflammatory model [Chia *et al* 2003b] to assess the direct effects of intra-

arterial TNF- α administration on t-PA release. We were able to induce local vascular inflammation with a rise in local plasma IL-6 concentrations to levels comparable to those seen in patients with unstable angina [Biasucci *et al* 1996], without evidence of a *systemic* inflammatory response or change in plasma t-PA or PAI-1 concentrations in the non-infused arm. This local vascular inflammation caused a slow onset and sustained increase in plasma t-PA concentrations that continued for at least 2 hours after cessation of the TNF- α infusion. This establishes that vascular inflammation directly causes endothelial t-PA release in man.

The mechanism of TNF- α induced t-PA release has not been established. Inflammatory cytokines, such as TNF- α , may cause t-PA release via induction of local thrombus formation, activation of specific cellular receptors or through generation of secondary mediators within the local vasculature. The former seems unlikely given that we observed no increase in prothrombin F1+2, a sensitive marker of *in vivo* thrombin generation [Paramo *et al* 2004]. Whilst it is likely that plasma t-PA concentrations are increased by inducing endothelial injury, smooth muscle cells, macrophages and monocytes also express t-PA mRNA following stimulation by inflammatory cytokines within atherosclerotic plaques [Lupu *et al* 1995] and could theoretically contribute to this fibrinolytic response.

5.5.1 EFFECTS OF TNF- α ON VASOMOTOR FUNCTION

Impaired vasodilator responses to acetylcholine [Suwaidi *et al* 2000; Heitzer *et al* 2001] and sodium nitroprusside [Heitzer *et al* 2001] in patients with cardiovascular risk factors predict an increased risk of adverse cardiovascular events. Previous

studies in healthy volunteers have shown acute systemic inflammation is associated with a transient impairment in vasomotor function [Hingorani *et al* 2000b]. We have now shown that, in patients with CHD, acute local vascular inflammation decreases the vasomotor response to both acetylcholine and nitroprusside. Acetylcholine is known to stimulate NO production via activation of eNOS and, together with the impaired nitroprusside response, these findings suggest that local arterial inflammation can decrease NO bioavailability. Interestingly, impaired vasodilator responses to both acetylcholine and sodium nitroprusside have been correlated with plasma TNF- α concentrations in patients with rheumatoid arthritis, a chronic inflammatory condition which is itself associated with an excess cardiovascular risk [Yki-Jarvinen *et al* 2003]. Furthermore, intra-arterial infusion of the free radical scavenger vitamin C restores FBF responses in patients with CHD and elevated serum levels of CRP [Fichtlscherer *et al* 2004].

Our results suggest that acute inflammation is associated with impaired NO-dependent smooth muscle relaxation in response to direct NO donors such as sodium nitroprusside or endogenously derived NO following stimulation of eNOS. Several lines of evidence support the contention that cytokines such as TNF- α may impair NO-dependent signalling. TNF- α decreases eNOS expression [Yoshizumi *et al* 1993] as well as increasing reactive oxygen species such as superoxide anion that rapidly inactivate NO and are directly cytotoxic to vascular tissues. TNF- α also increases plasma asymmetric dimethylarginine (ADMA), an endogenous inhibitor of eNOS that inhibits endothelium-dependent vasodilatation [Boger *et al* 1998]. Finally, inflammatory states may increase inducible nitric oxide synthase (iNOS) expression

which is associated with receptor uncoupling and endothelial dysfunction [Funakoshi *et al* 2002].

Bradykinin-induced vasodilatation was unaltered by pre-treatment with TNF- α . Previous work has suggested that NO contributes only a small proportion (~15%) to bradykinin-induced vasodilatation [O'Kane *et al* 1994; Honing *et al* 2000] and does not contribute to the mechanism of bradykinin-induced t-PA release [Brown *et al* 2000]. Moreover, in patients with vascular dysfunction, there may be an increased contribution of endothelium-derived hyperpolarising factor to smooth muscle vasorelaxation [Panza *et al* 1995] particularly if NO is consumed by free radicals generated by locally active inflammatory cells. These observations are consistent with our findings of impaired acetylcholine and sodium nitroprusside-induced vasodilatation but preserved bradykinin responses.

5.5.2 EFFECTS OF TNF- α ON ACUTE ENDOGENOUS FIBRINOLYSIS

Despite the presence of higher baseline plasma t-PA concentrations in patients with atherosclerosis [Lowe *et al* 2004], we have here shown that direct intra-arterial TNF- α infusion increases bradykinin-induced t-PA release in patients with stable CHD. Thus, although vasorelaxation was impaired, acute inflammation initiates a sustained increase in both basal and stimulated t-PA release. The mechanism of this effect is unknown but may involve direct endothelial injury, upregulation of t-PA synthesis or alterations in bradykinin receptor expression.

Under some circumstances, increases in t-PA may protect against the propagation of intravascular thrombosis and thereby avoid the development of an acute coronary syndrome. However, elevations in plasma t-PA concentrations may reflect more widespread endothelial dysfunction and a dominant pro-inflammatory vascular response that may overwhelm any locally protective pro-fibrinolytic effect. Indeed, the pro-fibrinolytic actions of vascular inflammation may potentiate degradation of extracellular matrix and aggravate plaque instability [Steins *et al* 1999]. The clinical outcome of acute vascular inflammation may, therefore, depend upon the relative balance between the protective antithrombotic actions and potential plaque destabilisation associated with increased vascular t-PA release.

In this study, all patients who received TNF- α and placebo infusion were already receiving secondary preventative therapy including aspirin and lipid-lowering medications. Although these may have influenced the vascular response to TNF- α , it was considered unethical to withhold these and, in clinical practice, a large proportion of patients presenting with acute coronary syndromes and raised inflammatory markers are already established on such therapies. As our study design was focused on the question of the link between vascular inflammation and t-PA release in patients with CHD, we did not include a control population of healthy subjects. However we have previously shown that intra-arterial TNF- α enhances endothelium-dependent t-PA release by a similar degree in younger healthy volunteers [Chia *et al* 2003b). Although we have again demonstrated that smokers have impaired endothelial responses including t-PA release [Newby *et al* 1999],

intra-arterial TNF- α increased plasma t-PA concentrations to a similar degree in both smokers and non-smokers with established CHD.

We have found that while TNF- α adversely affects NO-dependent vasodilatation, it enhances other protective mechanisms, such as the endogenous fibrinolytic capacity. This reflects the complex and pleiotropic nature of TNF- α which functions as part of the normal host surveillance mechanisms and response to tissue injury. Although we only determined the effect of acute vascular inflammation in twelve patients, our results may explain some of the contradictory findings of previous clinical studies. For example, in patients with heart failure, TNF- α antagonism causes marked improvements in endothelium-dependent vasodilatation [Fichtlscherer *et al* 2001] but has failed to demonstrate clinical benefit in randomised controlled trials [Mann *et al* 2004]. Thus, the benefits of restoring endothelium-dependent vasomotor function by TNF- α antagonism may be counterbalanced by inhibiting other potentially beneficial acute effects such as enhancing endogenous t-PA release.

In summary, we have shown that, in patients with stable CHD, plasma fibrinolytic factors are correlated with CRP, a sensitive and prognostically relevant marker of vascular inflammation. We have also demonstrated that acute vascular inflammation directly impairs vasomotor function whilst enhancing endothelial t-PA release. We suggest that the adverse prognosis associated with elevated plasma t-PA concentrations reflects a causative association with vascular inflammation and injury rather than representing a marker of occult plaque rupture.

CHAPTER 6

PHOSPHODIESTERASE TYPE 5 INHIBITION DOES NOT REVERSE ENDOTHELIAL DYSFUNCTION IN PATIENTS WITH CORONARY HEART DISEASE

Robinson SD, Ludlam CA, Boon NA, Newby DE.
Phosphodiesterase type 5 inhibition does not reverse endothelial
dysfunction in patients with coronary heart disease.
Heart 2006;**92**(2):170-6.

6.1 SUMMARY

Endothelial dysfunction is an independent predictor of cardiovascular events and may be due to a reduction in NO bioavailability. Phosphodiesterase inhibitors may augment NO-dependent pathways. We hypothesised that sildenafil citrate, a selective PDE 5 inhibitor, would improve endothelial vasomotor and fibrinolytic function in patients with CHD. Sixteen male patients with CHD and eight age-matched healthy men received intravenous sildenafil or placebo in a randomised double-blind placebo-controlled crossover study. Bilateral FBF and fibrinolytic parameters were measured using venous occlusion plethysmography and blood sampling in response to intra-brachial infusions of acetylcholine, substance P, sodium nitroprusside and verapamil. Mean arterial blood pressure fell during sildenafil infusion from (mean \pm SEM) 92 ± 1 to 82 ± 1 mmHg in patients and from 94 ± 1 to 82 ± 1 mmHg in controls ($p < 0.001$ for both). Sildenafil increased endothelium-independent vasodilatation with sodium nitroprusside ($p < 0.05$) but did not alter the blood flow response to acetylcholine or verapamil in patients or controls. Substance P caused a dose-dependent increase in t-PTA antigen concentrations ($p < 0.01$) that was unaffected by sildenafil in either group. Sildenafil does not improve peripheral endothelium-dependent vasomotor or fibrinolytic function in patients with CHD. Phosphodiesterase type 5 inhibitors are unlikely to reverse the generalised vascular dysfunction seen in patients with CHD.

6.2 INTRODUCTION

The endothelium plays an important role in the regulation of vascular function including local blood flow and endogenous fibrinolysis. Coronary heart disease and its risk factors, such as cigarette smoking, hyperlipidaemia and hypertension [Creager *et al* 1990; Celermajer *et al* 1993; Panza *et al* 1993] are associated with impaired endothelium-dependent vasorelaxation and reduced endothelial release of the endogenous fibrinolytic factor, t-PA [Newby *et al* 1999; Pretorius *et al* 2002]. These aspects of endothelial function are important since plasma fibrinolytic variables and endothelium-dependent vasodilatation independently predict future cardiovascular risk [Meade *et al* 1993; Suwaidi *et al* 2000].

Nitric oxide is a key factor linked to the beneficial protective effects of the endothelium and a decrease in NO bioavailability favours atherogenesis [Cayatte *et al* 1994]. Nitric oxide exerts many of its biological effects through generation of cGMP following activation of soluble guanylate cyclase. Phosphodiesterase type 5 inactivates cGMP within vascular smooth muscle and thus negatively regulates NO-mediated cellular actions [Wallis *et al* 1999]. Recently, highly selective PDE 5 inhibitors that prolong the action of cGMP and thereby enhance NO-mediated effects have become available for clinical use.

It has been suggested that the PDE 5 inhibitor, sildenafil citrate (Viagra[®], Pfizer Inc., New York), can improve endothelial vasomotor function in the peripheral circulation of healthy cigarette smokers [Kimura *et al* 2003] and the coronary circulation of

patients with CHD [Halcox *et al* 2002a]. Although we have previously demonstrated a link between NO and acute t-PA release [Newby *et al* 1998] the potential beneficial effects of sildenafil on t-PA and its inhibitor, PAI-1, are unknown.

We hypothesised that sildenafil would favourably alter endothelium-dependent vasomotor function and acute t-PA release in patients with stable CHD. If so, PDE 5 inhibitors might become a useful adjunctive therapy and confer secondary preventative benefits in patients with CHD.

6.3 METHODS

6.3.1 SUBJECTS

Sixteen male subjects with stable CHD and eight age-matched healthy control men participated in the study. The investigation was undertaken with the approval of the local Research Ethics Committee, the written informed consent of each subject, and in accordance with the principles outlined in the Declaration of Helsinki.

6.3.2 SUBJECTS WITH CORONARY HEART DISEASE

A history of CHD was confirmed by angiographic evidence of $\leq 50\%$ luminal stenosis of at least one major epicardial coronary vessel or a history of Q-wave MI. Nitrate medications were withdrawn for 48 hours prior to each visit and other medications were withheld on the morning of study. Patient exclusion criteria were significant cardiac failure, renal impairment, systolic blood pressure < 100 or > 190 mmHg and diabetes mellitus.

6.3.3 HEALTHY CONTROL SUBJECTS

Control subjects were healthy normotensive euglycaemic non-smokers without any history of cardiorespiratory or vascular disease and were not taking any regular medications. No subject had received sildenafil or other PDE inhibitor prior to, or during, participation in this study.

6.3.4 DRUGS

Substance P (Clinalfa AG, Switzerland), acetylcholine (Novartis UK Ltd), sodium nitroprusside (David Bull Laboratories, UK) and verapamil (Abbott UK Ltd) were administered following dissolution in 0.9% saline. Sildenafil and matched placebo (Pfizer Ltd, Sandwich, UK) were administered intravenously as a bolus and continuous infusion to achieve stable plasma concentrations equivalent to the peak concentration of a single 100 mg oral dose (pharmacokinetic data, Pfizer UK Ltd).

6.3.5 FOREARM STUDY PROTOCOL

Subjects were requested to abstain from alcohol for 24 hours and food, caffeine-containing drinks and tobacco for at least 4 hours before each study. All studies were carried out in a quiet temperature-controlled room maintained at 22-25°C. Subjects rested recumbent, strain gauges and cuffs were applied. The brachial artery of the non-dominant arm was cannulated with a 27-G needle, a venous cannula was inserted into the antecubital vein of each arm and into a dorsal foot vein for the administration of either intravenous sildenafil or matched placebo. Blood pressure and heart rate were monitored at intervals throughout the study in the non-infused arm using a semi-automated non-invasive sphygmomanometer. Mean arterial pressure was defined as the diastolic pressure plus a third of the pulse pressure.

6.3.6 STUDY DESIGN

Each subject attended at 09:00 hours on two separate occasions at least 2 weeks apart and received matched placebo and sildenafil in a randomised double-blind crossover design.

Saline was infused intra-arterially for the first 20 minutes to allow recording of resting FBF, blood pressure and heart rate. After this period, sildenafil or matched placebo was administered intravenously as a single 26.25 mg bolus over 5 minutes then as a continuous infusion of 10 mg/hr. Twenty minutes after commencing the sildenafil or placebo infusion, basal FBF was determined, and thereafter acetylcholine (5, 10, 20 $\mu\text{g}/\text{min}$), substance P (2, 4, 8 pmol/min), sodium nitroprusside (2, 4, 8 $\mu\text{g}/\text{min}$) and verapamil (10, 30, 100 $\mu\text{g}/\text{min}$) were infused intra-arterially for 6 minutes at each dose. Acetylcholine, substance P and sodium nitroprusside were given in a random order and separated by 20 minute saline washout periods, but because of its prolonged vasodilator action, verapamil was infused last. The order of the infusions was maintained constant for each subject across both visits.

6.3.7 STATISTICAL ANALYSIS

Data were examined, where appropriate, by ANOVA with repeated measures and two-tailed Student's *t*-test using GraphPad Prism Software (GraphPad Software, California, USA). All results are expressed as mean \pm SEM. Statistical significance was assigned at the 5% level. On the basis of a previous study [Newby *et al* 2002] the study had an 80% power to detect a 23% change in plasma t-PA concentrations and a 22% difference in FBF in CHD patients between sildenafil and placebo at the 5% level.

6.4 RESULTS

Most patients with CHD had a prior history of MI, hypertension and hyperlipidaemia (Table 6.1).

Reflecting concomitant therapy, mean resting heart rate (55 ± 1 versus 63 ± 2 beats/minute respectively, $p < 0.001$, unpaired t -test) and serum total cholesterol concentration (4.2 ± 0.2 versus 5.5 ± 0.2 mmol/L, $p < 0.001$) were lower in patients with CHD than controls. There were no differences in baseline MAP, resting heart rate, baseline FBF or haematocrit between the two study visits. Infusions were well tolerated and there were no serious adverse events. For technical reasons, 1 control subject was unable to complete both visits.

Table 6.1 Baseline characteristics

	Patients	Controls
Age, years	57±2	54±2
Body mass index, kg/m ²	27±1	27±1
Co-morbidity, n		
Myocardial Infarction	10	0
Hypertension	12	0
Diabetes mellitus	0	0
Previous hyperlipidaemia	15	0
Smoker / Non-smoker	1 / 15	0 / 8
Medical Therapy, n		
Aspirin	16	0
β-Adrenergic blockade	13	0
Calcium antagonist	3	0
Long acting nitrate/nicorandil	2	0
ACE inhibitor, AT II antagonist	5	0
Lipid-lowering therapy	16	0
Serum urea, mmol/L	5.5±0.3	5.1±0.4
Serum creatinine, µmol/L	92±3	95±4
Glucose, mmol/L	5.6±0.2	5.5±0.3
Total cholesterol, mmol/L	4.2±0.2	5.5±0.2*
HDL cholesterol, mmol/L	1.0±0.0	1.1±0.1
Triglycerides, mmol/L	1.7±0.2	1.2±0.2
Placebo visit		
Heart rate /min	55±1	61±3*
Mean arterial pressure, mmHg	95±2	95±4
Forearm blood flow, mL/100 mL/min		
Infused arm	2.5±0.2	2.2±0.2
Non-infused arm	2.3±0.2	2.1±0.4
Sildenafil visit		
Heart rate/min	55±1	65±3*
Mean arterial pressure, mmHg	95±2	91±3
Forearm blood flow, mL/100 mL/min		
Infused arm	2.5±0.2	2.6±0.3
Non-infused arm	2.5±0.2	2.4±0.3

All values are mean ± SEM. *p<0.001, unpaired *t*-test, patients *versus* controls.

ACE - angiotensin-converting enzyme; HDL - high density lipoprotein; SEM - standard error of mean.

6.4.1 HAEMODYNAMIC EFFECTS

Over the course of the study, the average MAP was lower during sildenafil than placebo infusion in patients with CHD (82 ± 1 *versus* 92 ± 1 mmHg, $p < 0.001$, unpaired *t*-test sildenafil *versus* placebo; Figure 6.1) and control subjects (82 ± 1 *versus* 94 ± 1 mmHg, $p < 0.001$, unpaired *t*-test). It returned to baseline after discontinuation of infusion (data not shown). There was a transient rise in heart rate following the sildenafil bolus in both groups (Figure 6.1 and data on file).

6.4.2 PLACEBO VISIT

Acetylcholine caused dose-dependent increases in FBF in both groups although this rise was significantly less in patients with CHD than controls ($p = 0.005$, ANOVA; Figure 6.2). There were no differences in FBF responses between the two groups during sodium nitroprusside and verapamil infusions (Figure 6.2). There were no significant changes in the non-infused FBF.

Figure 6.1 Heart rate (a) and MAP (b) during sildenafil (closed circles, solid line) or placebo (open circles, dashed line) infusion (shaded box) in patients with coronary heart disease.

* $p < 0.001$, ANOVA, sildenafil *versus* matched placebo; Control subject data on file ($p < 0.001$, ANOVA, MAP sildenafil *versus* matched placebo).

Bpm - beats per minute; MAP - mean arterial pressure.

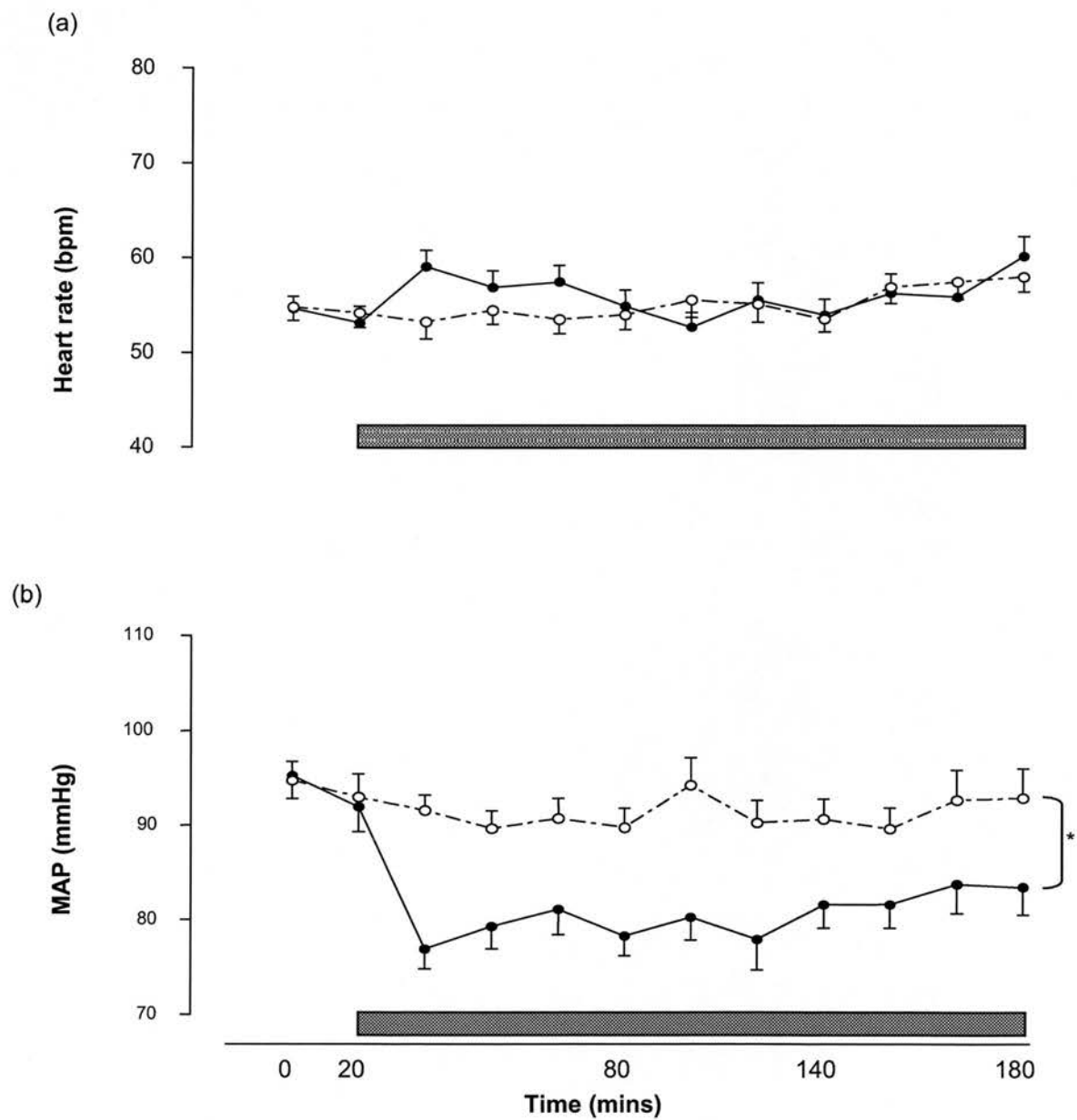
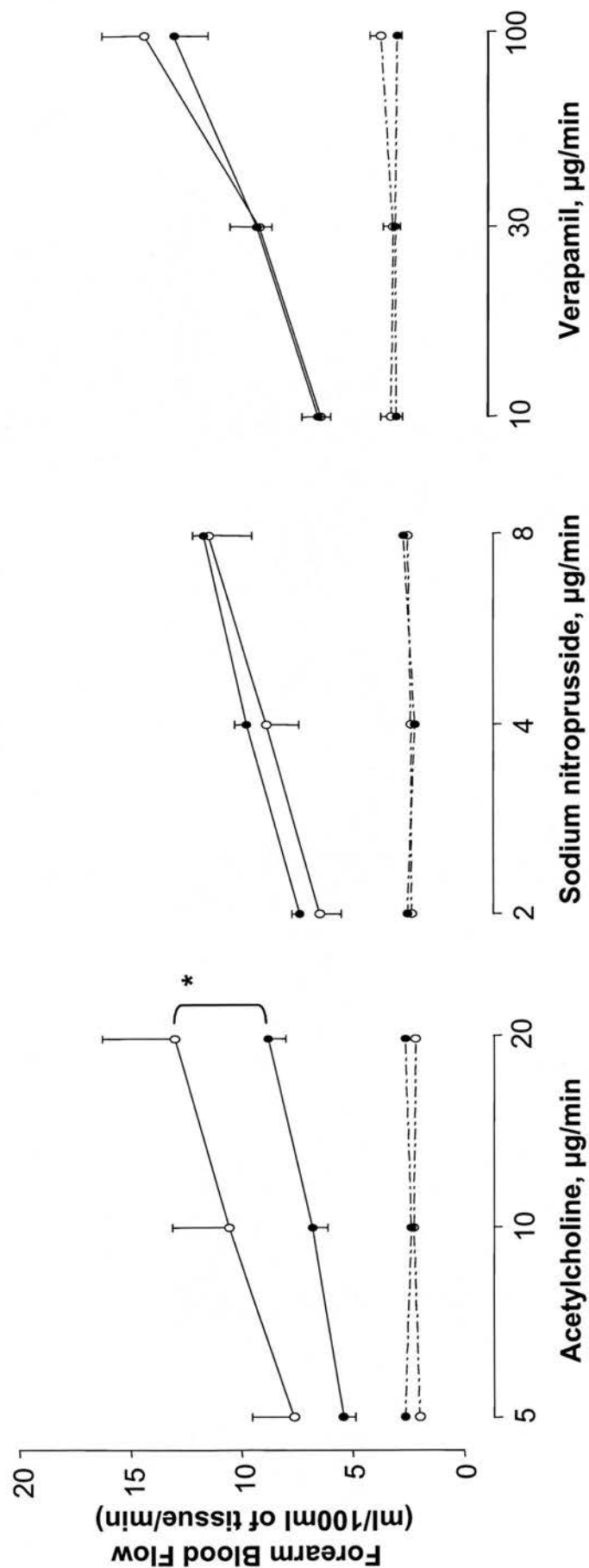


Figure 6.2 Infused (solid line) and non-infused (dashed line) forearm blood flow in patients with coronary heart disease (●) and controls (○) during intra-brachial acetylcholine (left), sodium nitroprusside (middle) and verapamil (right) with placebo infusion. $p < 0.001$, ANOVA, dose-response in infused arm; $*p = 0.005$, ANOVA; (●) CHD patients versus (○) controls.



6.4.3 SILDENAFIL THERAPY AND VASCULAR FUNCTION

Compared with placebo, administration of sildenafil caused no significant change in the infused FBF response during intra-arterial infusion of acetylcholine (at 20 $\mu\text{g}/\text{min}$, mean difference 0.1 mL/100 mL/min; 95% confidence intervals -0.2 to +0.4), substance P (at 8 pmol/min, mean difference 0.5 mL/100 mL/min; 95% confidence intervals 0.0 to +0.9) or verapamil (at 8 pmol/min, mean difference 0.3 mL/100 mL/min; 95% confidence intervals -0.1 to +0.7). However, sildenafil augmented the vasodilatation to sodium nitroprusside in both patients with CHD ($p < 0.05$, ANOVA; Figure 6.3) and control subjects ($p < 0.001$, ANOVA; Figure 6.4).

Figure 6.3 Infused (solid line) and non-infused (dashed line) forearm blood flow in patients with coronary heart disease during intra-brachial sodium nitroprusside (SNP; left), acetylcholine (middle) and verapamil (right) with sildenafil (●) and matched placebo (○) infusion. $p < 0.001$, ANOVA, dose-response in infused arm.; * $p < 0.05$, ANOVA; sildenafil (●) *versus* matched placebo (○).

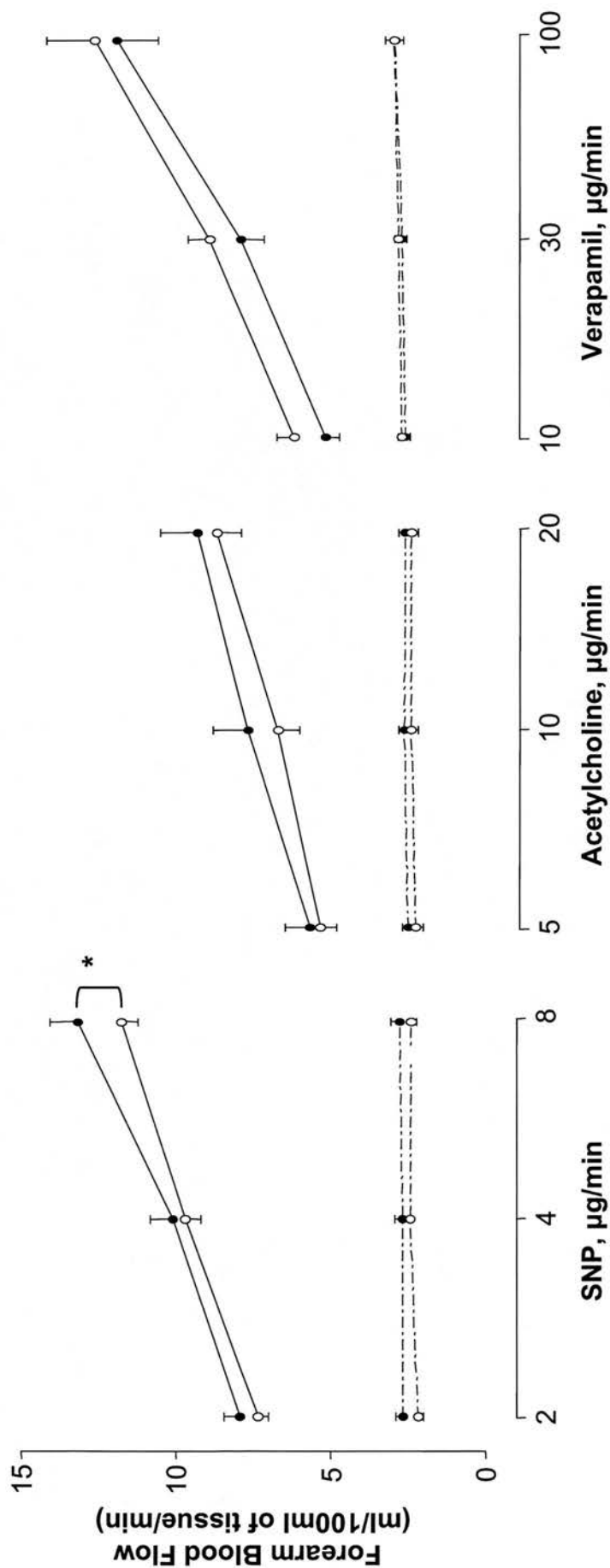
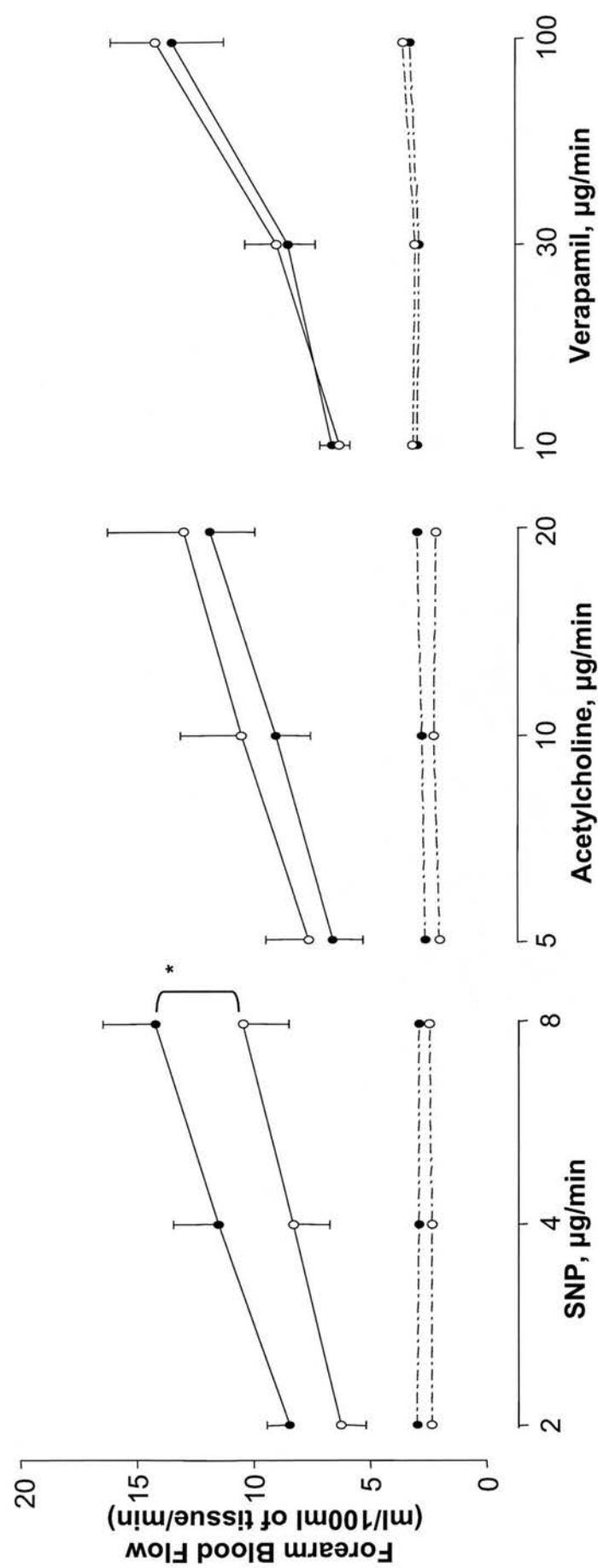


Figure 6.4 Infused (solid line) and non-infused (dashed line) forearm blood flow in healthy controls during intra-brachial sodium nitroprusside (SNP; left), acetylcholine (middle) and verapamil (right) with sildenafil (●) and matched placebo (○) infusion. $p \leq 0.01$, ANOVA, dose-response in infused arm; $p < 0.001$, ANOVA; sildenafil (●) *versus* matched placebo (○).



6.4.4 PLASMA FIBRINOLYTIC VARIABLES

Baseline plasma t-PA antigen concentrations were unchanged by sildenafil in either group (Table 6.2, Figure 6.5). Substance P caused a dose-dependent increase in plasma t-PA concentrations in both patients and controls ($p < 0.01$ for both, ANOVA; Table 6.2). There was no difference in substance P-induced increase in plasma t-PA concentrations during the sildenafil or placebo infusion (Table 6.2; at 8 pmol/min, mean difference -0.1 ng/mL; 95% confidence intervals -1.3 to +1.1) and there was no significant change in plasma PAI-1 concentrations throughout either study.

Figure 6.5 Infused (solid line) and non-infused (dashed line) FBF (right) and estimated net release of t-PA antigen (left) at baseline, during sildenafil (●) and matched placebo (○) infusion (shaded box), and subsequently with intra-brachial substance P (2, 4, 8 pmol/min) in patients with coronary heart disease.
 $p < 0.01$, ANOVA, dose-response for infused arm FBF and net t-PA release.
t-PA – tissue plasminogen activator; FBF – forearm blood flow.

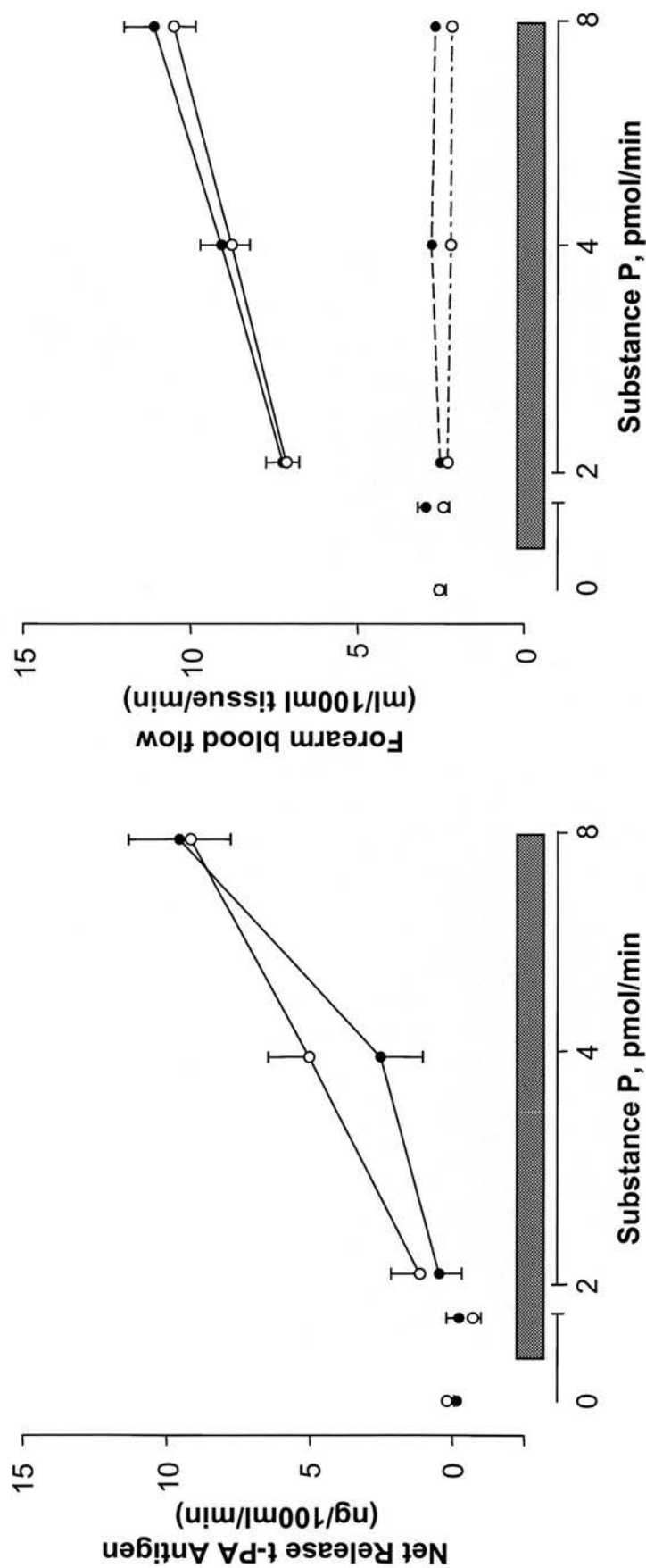


Table 6.2 Plasma tissue plasminogen activator (t-PA) and plasminogen activator inhibitor type 1 (PAI-1) concentrations at baseline and during sildenafil and matched placebo infusion in patients with coronary heart disease

*p<0.01, ANOVA for t-PA response; Control subject data on file (p<0.01, ANOVA for t-PA response).

Sildenafil/Placebo	0	Bolus	Continuous infusion (10mg/hr)		
Substance P dose, pmol/min			2	4	8
Placebo					
Plasma t-PA Antigen, ng/mL					
Infused arm	9.0±0.7	8.7±0.6	9.3±0.6	9.8±0.7	10.5±0.8*
Non-infused arm	9.0±0.6	9.1±0.6	9.1±0.6	8.9±0.6	9.1±0.6
Plasma PAI-1 Antigen, ng/mL					
Infused arm	46.8±6.9	43.1±6.0	-	-	34.4±5.4
Non-infused arm	45.2±7.1	45.5±7.6	-	-	36.4±6.3
Sildenafil					
Plasma t-PA Antigen, ng/mL					
Infused arm	9.0±0.6	8.7±0.6	9.1±0.6	9.4±0.6	10.5±0.7*
Non-infused arm	9.1±0.6	8.8±0.6	9.0±0.6	8.9±0.6	9.0±0.6
Plasma PAI-1 Antigen, ng/mL					
Infused arm	44.0±5.6	39.6±4.6	-	-	36.0±3.8
Non-infused arm	47.9±5.6	43.2±4.9	-	-	38.6±4.2

6.5 DISCUSSION

We have shown that sildenafil, a selective PDE 5 inhibitor, does not modify endothelium-dependent vasodilatation or acute t-PA release in men with stable CHD. However, sildenafil did augment the vasodilator effect of the exogenous NO donor, sodium nitroprusside. Thus, whilst our study confirms the well-described interaction of sildenafil with NO donors [Ishikura *et al* 2000; Kimura *et al* 2003) we have found no evidence to support the contention that PDE 5 inhibitors improve endothelium-dependent vasomotor or fibrinolytic function in patients with CHD.

Compared to matched controls, patients with CHD exhibited impairment of the endothelium-dependent responses to acetylcholine whilst having preserved vasodilator responses to the endothelium-independent agonists, sodium nitroprusside and verapamil. This prognostically significant impairment [Suwaidi *et al* 2000; Heitzer *et al* 2001] was evident in patients who were already receiving standard anti-anginal, anti-platelet and lipid-lowering therapies.

Sildenafil had no effect on flow-mediated dilatation of the brachial artery in patients with CHD [Halcox *et al* 2002a] and there are conflicting reports on the vasomotor responses of the coronary vessels to sildenafil. Herrmann and colleagues [Herrmann *et al* 2000] found no change in coronary artery diameter, blood flow or coronary vascular resistance whilst Halcox and colleagues [Halcox *et al* 2002a] reported enhanced coronary artery vasodilatation to acetylcholine. Unlike previous studies, we have, for the first time, used a more robust double-blind randomised placebo-controlled crossover study design and have shown that PDE 5 inhibition does not

alter either endothelium-dependent vasomotor or fibrinolytic function in patients with CHD or age-matched controls. Moreover, we used a bolus and continuous intravenous sildenafil infusion to minimise variations in plasma concentrations during the administration of each of the intra-arterial vasodilators. This is an important study consideration given the short half-life of sildenafil in humans.

We observed a decrease in MAP in both patients and controls during administration of sildenafil that presumably reflected an augmentation of the vascular effects of basal vascular NO release and is mediated through an increase in cGMP. Our findings are consistent with the published haemodynamic data in both healthy volunteers [Herrmann *et al* 2000] and patients with CHD [Jackson *et al* 1999; Herrmann *et al* 2000; Webb *et al* 2000] and confirm that we achieved a physiological effect with sildenafil infusion. The consistent vasodilatory response to the NO independent agonist, verapamil, makes it unlikely that administration of the PDE 5 inhibitor impaired vascular smooth muscle function or obscured potentially beneficial effects on endothelial function. Moreover, both acetylcholine and substance P produced similar, consistent and reproducible responses on both study days. This suggests that prolonging cGMP actions in patients with established atherosclerosis will not reverse endothelial dysfunction. As would be predicted from its mechanism of action, sildenafil augmented the responses to sodium nitroprusside, an exogenous NO donor, in both controls and CHD patients.

There are a number of potential reasons for the differences observed in the effect of sildenafil on the acetylcholine and sodium nitroprusside responses. The modest

decrease in acetylcholine-induced vasodilatation [Vallance *et al* 1989; Kamper *et al* 2002] seen following NOS inhibition suggests that non-NO-dependent pathways such as EDHF may predominate particularly in the presence of endothelial dysfunction [Bauersachs *et al* 1996; Katz and Krum 2001]. Indeed, differences in the relative contribution of endothelium-derived NO across vascular beds may explain some of the previously conflicting data on the vascular responses to sildenafil [Katz *et al* 2000; Halcox *et al* 2002a; Dishy *et al* 2004]. As well as endothelial dysfunction, atherosclerosis is associated with high levels of free radicals such as superoxide anion that rapidly react with NO to generate peroxynitrite, a powerful oxidant species that induces significant cellular damage and directly inhibits soluble guanylate cyclase [Vallance and Chan 2001]. Elegant studies in animals with specific knockouts of NO-dependent pathways [Ruetten *et al* 1999] also suggest that tissue specific downregulation of NO/cGMP, including cGMP-dependent protein kinase, may be an early feature of endothelial dysfunction in atherosclerotic conditions [Munzel *et al* 2003]. Phosphodiesterase type 5 inhibition would not be anticipated to influence changes in oxidative stress or directly affect cGMP independent NO molecular targets that contribute to endothelial dysfunction and atherogenesis. Therefore, the contrasting effects of sildenafil on acetylcholine and sodium nitroprusside-induced vasodilatation are likely to reflect a major dependence upon non-NO-mediated pathways, increased oxidative stress and decreased NO bioavailability associated with CHD.

Although the precise mechanism underlying acute t-PA release remains uncertain, several reports have previously suggested involvement of NO and cyclic nucleotides

regulated by PDEs. In animals, pentoxifylline and its analogues, non-selective PDE inhibitors, increased acute t-PA release [Tranquille and Emeis 1991b] and potentiated the effects of thrombolytic therapy [Ambrus *et al* 1994]. We and others have reported acute endothelial t-PA release during intra-arterial substance P [Newby *et al* 1997; Newby *et al* 1999], bradykinin [Brown *et al* 1999] and methacholine [Stein *et al* 1998] infusions as well as an inverse relationship between acute t-PA release and atherosclerotic plaque burden within the coronary circulation [Newby *et al* 2001]. In the present study, we have again demonstrated a rise in both plasma t-PA antigen concentrations and net t-PA release with local intra-arterial substance P infusion. However, infusion of sildenafil did not change basal plasma t-PA concentrations or substance P-induced t-PA release. It would, therefore, appear that enhancement of cGMP does not directly augment endothelial t-PA release in man.

Although we failed to show such a change in acute t-PA release using substance P, it remains possible that sildenafil could improve the response to other agonists such as bradykinin which causes B₂ receptor mediated prostacyclin and NO generation [Cockcroft *et al* 1994b]. However, the dominant mechanism of t-PA release with bradykinin appears to be NO and prostacyclin independent, and involves coupling of the B₂ receptor to the calcium-dependent G_q/phospholipase C-beta pathway [Brown *et al* 2000]. Some of the conflicting results on the effect of NOS inhibitors on t-PA release may suggest that there is physiological redundancy within the NO-dependent pathways that contribute to the regulation of acute t-PA release in man.

6.5.1 STUDY LIMITATIONS

In light of the haemodynamic changes seen in our study, intra-brachial infusion of sildenafil using subsystemic locally active doses would be one approach to assess the direct vascular actions of PDE 5 inhibition. However sildenafil is metabolised by the liver to an active metabolite that accounts for nearly half of its PDE inhibitory activity. Local intra-arterial infusion would not assess the action of this important metabolite. The effect of chronic PDE 5 inhibitor therapy is unclear and further studies involving females and subjects with diabetes mellitus would be of interest as these groups may show differences within NO-dependent pathways [Forte *et al* 1998; Brodsky *et al* 2001].

In summary, despite being highly effective in the management of erectile dysfunction, sildenafil does not modify endothelium-dependent vasomotor or fibrinolytic function in patients with CHD. Phosphodiesterase inhibitors have already shown promise as novel therapies in conditions such as chronic heart failure [Katz *et al* 2000] and pulmonary hypertension [Michelakis *et al* 2003] and these areas clearly warrant further research. However on the basis of our results, we believe that PDE 5 inhibitors are unlikely to reverse the generalised vascular dysfunction seen in patients with CHD.

CHAPTER 7

CONCLUSIONS AND FUTURE DIRECTIONS

7.1 SUMMARY OF THESIS FINDINGS

The endothelium plays a vital role in the regulation of blood flow, coagulation and fibrinolysis as well as mediating the response to tissue injury and inflammation. Disruption to these pathways is a critical factor in the development of atherosclerosis and coronary heart disease including clinical syndromes such as acute myocardial infarction. We have described a number of inter-related studies on factors affecting vascular function in a well characterised cohort of patients with stable CHD.

Our group had previously shown that cigarette smokers exhibited impaired fibrinolytic and vasomotor responses within both the forearm [Newby *et al* 1999] and coronary [Newby *et al* 2001] circulations. As smokers are at particular risk of coronary thrombosis, we hypothesised that endothelial fibrinolytic capacity would predict the future risk of atherothrombotic events in patients with CHD. We have for the first time shown that acute stimulated t-PA release from the forearm circulation is a powerful predictor of the risk of future atherothrombotic events. This validates the application of the forearm model to studying the pathophysiological changes observed in CHD and suggests that the acute endogenous fibrinolytic capacity is a novel bio-marker of future cardiovascular risk. Previous studies in healthy volunteers suggested that genetic variation within the t-PA locus affected basal t-PA release. However, we found no effect of a number of t-PA genetic polymorphisms on acute t-PA release in patients with established coronary disease. Inter-individual differences in t-PA release in subjects with CHD may therefore predominantly reflect the presence of environment factors such as cigarette smoking or the presence of as

yet unidentified genetic factors. Given the association between circulating levels of inflammatory markers and the risk of CHD, we determined the effect of an intra-arterial infusion of a pro-inflammatory cytokine on vascular function. We found that local intra-arterial inflammation impaired NO-dependent vasodilatation whilst augmenting endogenous t-PA release. This suggests a pathophysiological mechanism whereby circulating levels of inflammatory cytokines are directly related to plasma t-PA concentrations in subjects at risk of future cardiovascular events. Although impaired NO bioavailability appears to play a role in the expression of a pro-thrombotic endothelial phenotype, infusion of a highly selective PDE 5 inhibitor to try to prolong the biological actions of NO did not affect either endothelium-dependent vasodilatation or endogenous fibrinolysis in CHD patients.

7.2 ENDOTHELIAL FUNCTION AND RISK OF ATHEROTHROMBOTIC EVENTS

7.2.1 REGULATION OF VASCULAR TONE AND FUTURE CARDIOVASCULAR EVENTS

Measurement of vascular responses within the coronary circulation is of greatest relevance to understanding the mechanisms contributing to coronary thrombosis and MI. Assessing coronary blood flow responses is invasive and can only really be applied to subjects undergoing coronary angiography with its inherent risks. Despite these limitations, several studies have demonstrated that impaired endothelium-dependent vasodilatation within the coronary circulation is an adverse prognostic marker [Schachinger *et al* 2000; Suwaidi *et al* 2000; Halcox *et al* 2002b]. However, in only one of these studies [Halcox *et al* 2002b], was target vessel revascularisation excluded from the composite endpoint of cardiovascular events. Including revascularisation procedures as an endpoint is contentious as it may be influenced by non-biological factors which are not directly due to the pathogenesis of coronary atherothrombosis and endothelial dysfunction [Widlansky *et al* 2003].

Although therapeutic interventions may lead to improvements in endothelium-dependent vasomotor responses, this does not necessarily equate to a reduction in clinical events. A number of trials of hormone replacement therapy demonstrated beneficial effects on coronary and peripheral vascular tone, yet primary and secondary preventative trials have been proven negative [Ganz 2002; Ganz and Vita 2003]. This highlights the difficulty in using only the vasodilator response as a surrogate marker of endothelial function. The endothelium continuously releases

paracrine factors, such as NO and prostacyclin, that not only modulate vascular tone but also influence local platelet activation and aggregation. We [Robinson *et al* 2006b] have recently shown that those patients with the lowest endothelium-dependent vasodilatation have the highest levels of circulating platelet-leukocyte aggregates which are a sensitive and prognostically useful marker of *in vivo* platelet activation [Michelson *et al* 2001; Sarma *et al* 2002]. These novel findings may be relevant in understanding the link between impaired endothelium-dependent vasodilatation and the risk of acute thrombotic-related cardiovascular events.

7.2.2 ACUTE T-PA RELEASE FROM THE FOREARM CIRCULATION PREDICTS FUTURE ATHEROTHROMBOTIC EVENTS

The forearm circulation is more accessible than the coronary bed, though as it is relatively protected from the development of atheroma, its applicability to the study of CHD has been questioned. Our group had previously shown that cigarette smokers, a group at particular risk of coronary thrombosis, exhibit impaired acute t-PA release in response to substance P infusion within both the forearm [Newby *et al* 1999] and coronary [Newby *et al* 2001] circulations. Here we have extended these observations and shown that acute stimulated t-PA release from the forearm circulation predicted the future risk of cardiovascular death, MI, CVA, and emergency hospitalisation for myocardial ischaemia. This association was demonstrated in a cohort of patients with angiographically proven coronary disease and confirms the validity of using the forearm model and acute t-PA release as a novel bio-marker of vascular risk. We specifically excluded events solely related to revascularisation procedures from our pre-defined endpoint hypothesising that

impaired t-PA release would be of greatest relevance in predicting thrombotic-related clinical events. However, impaired t-PA release may increase plaque growth as incomplete thrombus resolution is known to stimulate smooth muscle migration and plaque expansion [Davies 2000b]. Future prospective studies examining the relationship between acute t-PA release and the progression of coronary atheroma in addition to future atherothrombotic events would be of interest.

7.3 TISSUE PLASMINOGEN ACTIVATOR GENOTYPE AND ACUTE T-PA RELEASE

Our interest in assessing the importance of t-PA genotype in CHD patients arose from previous work suggesting several polymorphisms of t-PA gene contributed to inter-individual differences in basal t-PA release in healthy subjects [Jern *et al* 1999; Ladenvall *et al* 2001]. However, we have found no evidence of a significant effect of the *Alu* Ins/Del, -7,351 C/T, 20,099 T/C or 27,445 T/A polymorphisms on either plasma concentrations, or acute release, of t-PA in a cohort of subjects with stable CHD. This novel finding is in keeping with a more recent study of 240 Swedish subjects which found no association between the most common t-PA haplotypes and plasma levels of fibrinolytic factors [Ladenvall *et al* 2003]. As previous estimates have suggested heritability for plasma levels of fibrinolytic factors is in the order of 30-60% [Souto *et al* 2000; de Lange *et al* 2001], it is possible that other genes e.g. PAI-1 4G/5G polymorphism [Henry *et al* 1998] may contribute to inter-individual differences in endogenous fibrinolytic capacity.

7.3.1 GENOTYPE-ENVIRONMENT INTERACTIONS AND CARDIOVASCULAR RISK PHENOTYPE

The overall genetic contribution to CHD is presumably due to a combination of several gene-gene and gene-environment interactions. From this work we cannot ascertain whether t-PA genotype may contribute to the initiation of endothelial dysfunction or expression of a pro-atherothrombotic phenotype in healthy subjects, or in the presence of other vascular risk factors. As in previous studies, cigarette smoking was associated with significantly impaired acute t-PA release [Newby *et al* 1999; Newby *et al* 2001; Pretorius *et al* 2002] and endothelium-dependent vasodilatation [Celermajer *et al* 1993]. This suggests environmental factors such as smoking, rather than t-PA genotype are the predominant determinant of fibrinolytic function in patients with established coronary disease. Although we found no interaction between cigarette smoking and t-PA genotype in a post-hoc analysis, it is possible that subject numbers may have been insufficient to adequately assess the relationship between t-PA genotype, cigarette smoking and t-PA release.

7.4 INFLAMMATION DIRECTLY AFFECTS FIBRINOLYTIC FUNCTION IN PATIENTS WITH CORONARY HEART DISEASE

Acute experimental inflammation, induced by typhoid vaccination in healthy volunteers, is associated with transient impairments in vascular function [Hingorani *et al* 2000b; Chia *et al* 2003a]. This effect is, in part, mediated by the genesis of cyclooxygenase activity as it is prevented by systemic, but not local, aspirin therapy [Kharbanda *et al* 2002]. Systemic inflammation will have biological actions on a

diverse range of tissues stimulating the release of secondary mediators from organs such as the liver or activation of neurohormonal reflexes which may alter t-PA release [Jern *et al* 1994; Bjorkman *et al* 2003]. Using a model of acute local vascular inflammation, we have confirmed previous findings in healthy volunteers [Bhagat and Vallance 1997; Chia *et al* 2003b], and extended these observations to show that in subjects with established atherosclerosis, acute local arterial inflammation impairs NO-dependent vasodilatation whilst increasing t-PA release.

7.4.1 ARTERIAL INFLAMMATION DIRECTLY INFLUENCES TISSUE PLASMINOGEN ACTIVATOR RELEASE

These novel results suggest a rationale whereby increased plasma t-PA concentrations are associated with the risk of future atherothrombotic events [Hamsten *et al* 1985; Jansson *et al* 1993; Meade *et al* 1993; Ridker *et al* 1993; Thompson *et al* 1995; Thogersen *et al* 1998]. The increase in basal and *regulated* t-PA release suggests that inflammation-induced t-PA release is not simply a non-specific marker of vascular injury and loss of endothelial integrity. It is possible that acute arterial inflammation leads to an ‘anti-thrombotic’ pro-fibrinolytic milieu to counter a ‘pro-thrombotic’ reduction in NO-dependent vasodilatation and local blood flow. As plasminogen activator mRNA expression is greatest in advanced macrophage and lipid-rich plaques [Lupu *et al* 1995; Steins *et al* 1999], increased levels of pro-inflammatory cytokines might promote rupture of unstable ‘t-PA rich’ plaques with a risk of platelet-rich thrombus formation and potentially arterial occlusion. Although unproven, this hypothesis would also be in keeping with the

observation whereby higher circulating concentrations of inflammatory cytokines and t-PA predict an increased risk of future cardiovascular events.

7.4.2 VASCULAR INFLAMMATION AND NITRIC OXIDE

We have demonstrated that acute arterial inflammation impairs NO-dependent vasodilatation in patients with CHD. Previous studies of acute experimental inflammation in healthy volunteers [Hingorani *et al* 2000b; Kharbanda *et al* 2002; Chia *et al* 2003a; 2003b] may not be directly comparable to those in patients with stable CHD who exhibit evidence of impaired endothelium-dependent vasodilation and increased basal t-PA concentrations reflecting pre-existing vascular injury and endothelial dysfunction. In patients with rheumatoid arthritis, an inflammatory condition associated with an increased risk of cardiovascular disease, blunted NO-dependent vasodilator responses parallel evidence of inflammatory disease activity [Bergholm *et al* 2002; Yki-Jarvinen *et al* 2003]. Furthermore, in patients with autoimmune vasculitis, administration of anti-TNF- α therapy is associated with augmentation of the endothelium-dependent vasomotor responses [Booth *et al* 2004]. Our current findings in patients with CHD are thus in keeping with those of other inflammatory conditions and suggest that disruption to the normal physiological NO signalling pathways and impaired NO bioavailability are a critical factor in inflammation-induced vascular dysfunction. The relative expression and activity of inducible and constitutive eNOS, local levels of oxidative stress and free radicals likely determine the summative effect of an inflammatory stimulus on the vasculature.

7.5 PHOSPHODIESTERASE TYPE 5 INHIBITION AND ENDOTHELIAL FUNCTION

The vascular endothelium appears directly sensitive to each of the known atherogenic risk factors with loss of endothelially-derived NO appearing to be a critical factor in the propensity for vasoconstriction, thrombosis, inflammation and cellular proliferation. We hypothesised that sildenafil, a highly selective PDE 5 inhibitor, might restore normal endothelial function by prolonging the cellular actions of the downstream signalling molecule of NO. This was, in part, based on previous work demonstrating that the L-arginine:NO pathway was involved in the regulation of t-PA release [Newby *et al* 1998] and that non-selective PDE inhibitors potentiate the effect of exogenous thrombolytic therapy [Ambrus *et al* 1994].

Phosphodiesterase isoenzyme type 5 inhibitors have been shown to be highly efficacious in the treatment of erectile dysfunction [Goldstein *et al* 1998], eliciting arterial smooth muscle relaxation in response to increased cGMP concentrations. Recently, sildenafil was reported to augment forearm vasomotor responses in young healthy subjects including asymptomatic smokers [Kimura *et al* 2003] though this is not a consistent finding [Dishy *et al* 2004]. Despite confirming earlier reports [Jackson *et al* 1999; Herrmann *et al* 2000; Webb *et al* 2000] of a consistent and reproducible effect on augmenting basal NO release and reduced vessel tone, prolonging the biological actions of cGMP did not change acute t-PA release nor endothelium-dependent vasodilatation in patients with CHD or age-matched healthy controls.

Atherogenic risk factors such as free fatty acids and cigarette smoking increase oxidative stress and the production of free radicals such as superoxide. Superoxide rapidly deactivates NO [Gryglewski *et al* 1986] to produce peroxynitrite [Beckman *et al* 1990] which is cytotoxic and directly inhibits soluble guanylate cyclase. If levels of oxidative stress are high, therapeutic intervention with PDE 5 inhibitors may not be able to circumvent the rapid deactivation of NO before it reaches its molecular target. Indeed, oral administration of ascorbic acid does not increase t-PA release in healthy volunteers nor blunt the increased fibrinolytic response to acute hyperhomocysteinaemia [Labinjoh *et al* 2001b].

7.5.1 THERAPEUTIC USES OF PHOSPHODIESTERASE TYPE 5 INHIBITORS

In the recently published, African-American Heart Failure Trial (A-HeFT), combined administration of a NO stimulator and an antioxidant (isosorbide dinitrate and hydralazine) produced significant reductions in cardiac death, MI and risk of developing symptomatic heart failure [Taylor *et al* 2004]. This dual therapeutic strategy may better address the altered NO oxidation balance fundamental to the failing cardiovascular system [Hare 2004]. The combination of a PDE 5 inhibitor and a free radical scavenger might overcome the limitations of our PDE 5 monotherapy and yield clinically meaningful improvements in vascular function in patients with CHD. Although we did not observe any change in vasomotor or fibrinolytic function in CHD patients with sildenafil, PDE 5 inhibitors may have important effects on platelet function [Halcox *et al* 2002a] with the combination of sildenafil and aspirin reducing coronary thrombosis and *ex vivo* platelet aggregation more than aspirin

alone in a canine model of coronary obstruction [Lewis *et al* 2006]. In keeping with its effects on vascular tone [Webb *et al* 2000], we have recently demonstrated that the platelet anti-aggregatory effect of sildenafil appears to be greatest in the presence of NO donors [Gudmundsdottir *et al* 2005].

The high concentration of PDE 5 within the pulmonary circulation suggests that PDE 5 inhibitors might be a useful adjunctive therapy for the treatment of pulmonary hypertension (PHT). Early clinical trials of sildenafil have shown encouraging benefits in patients with primary and secondary PHT [Michelakis *et al* 2002; Michelakis *et al* 2003], conditions usually associated with high morbidity and mortality. Sildenafil has also been shown to improve brachial reactivity [Katz *et al* 2000] and exercise capacity in patients with congestive cardiac failure [Bocchi EA 2002]. There are large numbers of subjects who might benefit from these areas of ongoing research on the use of PDE 5 inhibitors in the management of symptomatic PHT and heart failure.

7.6 FUTURE DIRECTIONS

This body of work has examined factors modifying the acute fibrinolytic capacity and shown that this is a critical marker of endothelial function which predicts future vascular risk. Given the central role of inflammation and t-PA release in the initiation, progression and the final common pathway of atherothrombosis, a series of further studies are suggested.

7.6.1 ENDOGENOUS FIBRINOLYTIC FUNCTION AND FUTURE ATHEROTHROMBOTIC EVENTS

We have shown that, in patients with stable CHD, the acute endogenous fibrinolytic capacity is a powerful predictor of future atherothrombotic events. This relationship was demonstrated in subjects receiving secondary preventative medication including anti-platelet and lipid-lowering therapies. Further work on the relationship between acute stimulated t-PA release and future cardiovascular events in apparently healthy subjects, including asymptomatic cigarette smokers, would be of great interest. As the expected cardiovascular event rate would be lower in such a cohort, increased subject numbers and duration of follow-up would be necessary. As coronary t-PA release is inversely related to coronary plaque volume [Newby *et al* 2001], additional work examining the association between acute stimulated t-PA release, inflammatory bio-markers and serial assessment of the progression of coronary atheroma should be considered. This may yield a better understanding of factors involved in the progression of atherosclerosis and identify subjects who would benefit from more intensive risk factor modification or pharmacological intervention before the onset of symptomatic coronary disease.

7.6.2 ENDOTHELIAL NITRIC OXIDE SYNTHASE POLYMORPHISM AND VASCULAR FUNCTION IN PATIENTS WITH CORONARY HEART DISEASE

Three distinct isoforms of NOS exist. In the vasculature, type III or eNOS is responsible for the generation of NO and the regulation of vascular tone [Vallance *et al* 1989] and blood pressure [Haynes *et al* 1993]. Several eNOS polymorphisms have been described but of particular interest is the SNP at position 894 of exon 7

[Hingorani 2000a]. This coding sequence polymorphism predicts a Glu²⁹⁸Asp amino acid substitution with a change in codon 298 from glutamic to aspartic acid. This might alter the mature protein structure and enzyme activity with some [Tesauro *et al* 2000], but not all [Fairchild *et al* 2001], authors reporting the eNOS Asp variant is more susceptible to proteolytic cleavage.

The allelic frequency of this Glu²⁹⁸→Asp SNP is ~30% in control populations but is significantly higher in patients with coronary artery disease at ~ 50% [Hingorani *et al* 1999]. The eNOS Asp variant has been associated with functional alterations in endothelium-dependent regulation of vascular tone which are most marked in cigarette smokers [Leeson *et al* 2002]. However, no assessment was made of the acute fibrinolytic function of the endothelium. Given that both NOS inhibition [Newby *et al* 1998] and cigarette smoking [Newby *et al* 1999; Newby *et al* 2001] reduce acute t-PA release *in vivo* in man, there is a potential significant interaction between the Glu²⁹⁸→Asp polymorphism and cigarette smoking. This hypothesis is being addressed in a British Heart Foundation Project (FS/2001047) with preliminary results suggesting that the eNOS Glu²⁹⁸Asp variant does not significantly affect vasomotor or fibrinolytic function in patients with CHD [Robinson *et al* 2006d].

7.6.3 ANTI-CYTOKINE THERAPY IN PATIENTS WITH CORONARY HEART DISEASE

The clinical sequelae resulting from arterial inflammation are likely dependent on the relative balance of its effects on vascular tone, coagulation and fibrinolysis. Clinical trials of anti-cytokine therapies in patients with advanced heart failure suggest little prognostic or symptomatic benefit [Mann *et al* 2004] despite showing improvements

in endothelium-dependent responses [Fichtlscherer *et al* 2001]. Although considered a pro-inflammatory cytokine, the biological effects of TNF- α depend upon the molecular target, the state of cellular activation, and the local concentrations of the cytokine [Henriksen and Newby 2003]. Whilst brief local infusion of TNF- α impairs vasodilator responses [Bhagat and Vallance 1997; Chia *et al* 2003b, Robinson *et al* 2006a] more prolonged infusion results in venodilatation [Simper *et al* 1995], and at higher doses endotoxin causes hypotension [Suffredini *et al* 1989]. In keeping with this, clinical trials suggest greater doses [Chung *et al* 2003], or more prolonged duration of anti-cytokine therapy [Mann *et al* 2004] may be harmful. Non-specific anti-cytokine therapy may be insufficient to disrupt the network of pro-inflammatory mediators e.g. interleukin-1 and interleukin-6, and might impair the action of anti-inflammatory 'athero-protective' cytokines such as interleukin-10 [Heeschen *et al* 2003].

Unstable angina is associated with acute inflammation, endothelial dysfunction [Fichtlscherer *et al* 2000] and elevated plasma TNF- α concentrations are predictive of an adverse prognosis and recurrent coronary events [Ridker *et al* 2000a]. These findings suggest that TNF- α antagonism may provide a novel approach to normalise the inflammation and endothelial dysfunction associated with acute unstable angina. We are currently conducting a study to assess the effect of Etanercept (Enbrel), a recombinant human TNF receptor p75-Fc fusion protein that acts as a competitive inhibitor of TNF- α , on endothelial and fibrinolytic function in patients with acute coronary syndromes.

7.6.4 PARTICULATE AIR POLLUTION AND CARDIOVASCULAR DISEASE

Air pollution is now recognised as a significant cause of cardiovascular morbidity and mortality [Dockery *et al* 1993; Anderson *et al* 1996]. Rates of myocardial ischaemia, heart failure and death are all increased following both short- [Katsouyanni *et al* 2001; Pekkanen *et al* 2002] and long-term [Clancy *et al* 2002; Pope *et al* 2004] exposure to air pollution. The mechanism and components of air pollution responsible for the adverse cardiovascular effects are unknown but small combustion-derived particles are suspected to be the major cause.

Within this thesis, and in previous work [Newby *et al* 1999; Newby *et al* 2001; Pretorius *et al* 2002], reference has been made to the importance of cigarette smoking in mediating vascular dysfunction and impaired endogenous fibrinolysis. Combustion products and inhaled particulate matter (PM) are common to environmental air pollution and cigarette smoking, and both induce an inflammatory response involving increased oxidative stress [Donaldson *et al* 2003]. Given that cigarette smoking and air pollution share common toxicological properties, we wish to investigate the vascular and inflammatory effects of air pollution and its constituents.

A series of human studies are proposed to determine vascular function, endogenous fibrinolytic and inflammatory activity following a rigorously controlled PM exposure. Initially we will study healthy subjects and thereafter patients with stable CHD. Experimental studies of subjects with established CHD are vital as it has been suggested that they may be more susceptible to the effects of ambient pollution

[Brook *et al* 2004]. Two exposure systems will be utilised: (1) *Filtered Diesel Exhaust Exposure* in collaboration with Professor Thomas Sandström at the University of Umeå, Sweden who has extensive experience of human studies using this model [Rudell *et al* 1994; Salvi *et al* 1999]; (2) *Environmental Concentrated Ambient Air Particles* (CAPs) in collaboration with Dr Flemming Cassee from the National Institute of Public Health (RIVM) Netherlands. Using these complementary systems, the effect of filtered clean air or PM (dilute diesel exhaust or CAPs) on vascular function will be assessed in a series of double-blind randomised crossover exposures. We will assess vascular function and inflammatory markers before, and 6 to 8 hours after, the controlled exposure to coincide with the peak response to airborne pollution [Salvi *et al* 1999] and *s. typhi* vaccination [Hingorani *et al* 2000b; Kharbanda *et al* 2002; Chia *et al* 2003a]. The subjects in this study will be exposed to concentrations of particles that do not exceed what they might encounter in a polluted urban environment on a smoggy day within an urban environment [UN Environment Program and WHO Report 1994].

These series of studies should provide important information about the cardiovascular impact of air pollution relevant to both the general population and patients with CHD. Moreover, if the detrimental component can be identified, more effective pollution control strategies could be implemented to reduce the risk for susceptible individuals. This proposal will also guide future research into the benefits of anti-inflammatory therapy in susceptible individuals unavoidably exposed to the currently increasing levels of urban air pollution. The effect of air pollution on

vasomotor and fibrinolytic function is the subject of an on-going British Heart Foundation Research Project (03/017/15071; Dr Nicholas Mills).

7.7 CLINICAL PERSPECTIVE

The presence of obstructive coronary atheroma and chronic cigarette smoking are associated with impaired release of t-PA from the endothelium [Newby *et al* 2001]. Defective endogenous fibrinolysis may underlie the increased risk of acute coronary thrombosis and sudden death associated with cigarette smoking [Burke *et al* 1997; Doll *et al* 2004]. The invasive nature of measuring coronary t-PA release means it can only really be undertaken in subjects undergoing coronary angiography limiting its widespread applicability as a bio-marker of vascular function.

We have shown that a reduction in acute t-PA release from the forearm circulation predicts the risk of future atherothrombotic events in patients with established CHD. This was the first longitudinal study to assess the relationship between future vascular events and endogenous t-PA release. This suggests that the forearm model may be used to examine the perturbations in vascular function observed in patients with CHD. A more detailed characterisation of the cellular pathways regulating t-PA secretion will provide a basis for the investigation of the mechanisms of impaired secretion. The pleiotropic effects of vascular inflammation on vasomotor and fibrinolytic function [Hingorani *et al* 2000b; Kharbanda *et al* 2002; Chia *et al* 2003a; 2003b; Robinson *et al* 2006a] suggests that the term ‘endothelial dysfunction’ does not encompass a uniform response to vascular injury.

Although assessment of t-PA release is unlikely to become part of routine clinical cardiovascular risk assessment, the findings discussed within this thesis support the hypothesis that reduced t-PA release is of pathophysiological significance. For example, angiotensin-converting enzyme inhibitors potentially increase stimulated t-PA release within the forearm [Witherow *et al* 2002; Cruden *et al* 2005] and coronary [Minai *et al* 2001; Matsumoto *et al* 2003] circulations. This augmentation of the endogenous fibrinolytic capacity might underlie the consistent reduction in thrombotic events including acute MI and CVA observed in several randomised clinical trials of ACE inhibitors [Rutherford *et al* 1994; Yusuf S *et al* 2000; Fox KM *et al* 2003].

The future development, and use, of specific therapeutic interventions to increase endogenous t-PA release in CHD patients might have secondary preventative benefits such as reducing the risk of future MI and the tragic haemorrhagic complications which can ensue following the administration of exogenous fibrinolytic agents. Further work is also needed to establish whether primary preventative strategies designed to augment endogenous fibrinolytic capacity might delay or prevent the onset of symptomatic coronary atheroma in at risk subjects.

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ORIGINAL ARTICLE

Tissue plasminogen activator genetic polymorphisms do not influence tissue plasminogen activator release in patients with coronary heart disease

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Summary. *Objectives:* To determine if polymorphisms of the tissue plasminogen activator (*t-PA*) gene influence acute endogenous t-PA release in patients with coronary heart disease (CHD). *Methods:* Forearm blood flow and plasma t-PA concentrations were measured in response to intra-brachial infusion of substance P and sodium nitroprusside in 96 patients with stable CHD. Genotyping was performed using a *Taqman* polymerase chain reaction assay specifically designed to detect the polymorphisms of interest: (i) *Alu*-repeat insertion/deletion sequence; (ii) C → T substitution in an upstream enhancer region (–7351 C/T); (iii) T → C in exon 6 (20 099 T/C); and (iv) T → A (27 445 T/A) in intron 10. *Results:* Substance P and sodium nitroprusside caused dose-dependent increases in forearm blood flow in all patients ($P < 0.001$ for all) that were independent of the four genetic polymorphisms. Similarly, there were no differences in basal plasma t-PA antigen concentrations or net t-PA release between genotypes. Compared to non-smokers, smokers exhibited impaired substance P-induced vasodilatation ($P < 0.001$) and t-PA release ($P = 0.05$). *Conclusions:* Despite confirming our previous findings in cigarette smokers, we have found no effect of polymorphisms of the *t-PA* gene on two complementary aspects of endothelial function. We conclude that genetic variation of the t-PA locus is unlikely to have a major influence on acute t-PA release in subjects with established CHD.

Keywords: coronary heart disease, endothelial function, fibrinolysis, genetic polymorphisms.

Introduction

Coronary heart disease (CHD) and its risk factors, such as cigarette smoking, hyperlipidemia and hypertension, are associated with impaired endothelium dependent vasorelaxation [1–3] and tissue plasminogen activator (t-PA) release [4–6]. These aspects of endothelial function are important as plasma fibrinolytic variables and endothelium-dependent vasodilatation independently predict future cardiovascular risk [7,8].

Genetic factors influence plasma concentrations of a number of hemostatic and fibrinolytic factors including t-PA, plasminogen activator inhibitor type 1 (PAI-1) and fibrinogen [9]. A common intronic polymorphism of the *t-PA* gene is generated by the insertion of a 311 base pair *Alu*-repeat sequence [10]. Although not a consistent finding in all populations [11], there is an independent association between the insertion (II) genotype and the future risk of myocardial infarction (MI): a relative risk of 2.24 in comparison with the deletion (DD) genotype [12]. This *Alu* intronic sequence is now known to be in linkage disequilibrium (LD) with three single nucleotide polymorphisms associated with t-PA release, within the upstream enhancer region (7351 C → T), in exon 6 (20 099 T → C) and within intron 10 (27 445 T → A) [13]. The C → T substitution within the enhancer region disrupts a binding site for the Sp1 transcription factor and appears to be functionally important [13,14] with TT homozygotes having an increased risk of both MI [15] and lacunar stroke [16] compared to CC homozygotes.

Genetic differences in t-PA release may alter the fibrinolytic balance predisposing to local thrombus formation and an increased likelihood of adverse athero-thrombotic events. Jern and colleagues previously demonstrated a link between t-PA release and both the *Alu* insertion/deletion and –7351 C/T enhancer polymorphisms in healthy volunteers [13,17]. However the functional importance of these genetic variants in patients with established CHD is unknown. We hypothesized that polymorphisms of the *t-PA* gene may influence endothelial vasomotor and fibrinolytic function in patients with stable CHD.

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Methods

Subjects

We recruited patients with CHD confirmed by angiography (defined as > 70% luminal stenosis of at least one major epicardial coronary vessel) or a history of Q-wave MI. All patients had stable anginal symptoms and had not undergone coronary revascularization within the preceding 3 months. Exclusion criteria were significant cardiac failure, renal impairment, systolic blood pressure < 100 or > 190 mmHg, impaired fasting glucose (> 7 mmol L⁻¹) or prior diagnosis of diabetes mellitus. Self-reported smoking status was confirmed using plasma cotinine levels [18]. All studies were undertaken with the approval of the local Research Ethics Committee, the written informed consent of each subject, and in accordance with the Declaration of Helsinki.

Venous sampling and assays

Venous blood was collected into tubes containing acidified buffered citrate (for t-PA), trisodium citrate (for PAI-1 and cotinine), potassium ethylene diamine tetraacetic acid (for DNA extraction) and serum gel tubes (for C-reactive protein; CRP). Platelet-free plasma, serum and whole blood (DNA) were stored at -80 °C before assay. Plasma t-PA and PAI-1 antigen concentrations were determined using enzyme-linked immunosorbent assays (Coaliza[®]; Chromogenix, Mölndal, Sweden) [5] with intra-assay and inter-assay coefficients of variation of 7.0% and 5.5%, and 4.0% and 7.3% for t-PA and PAI-1 antigen, respectively [19]. All fibrinolytic assays were performed in duplicate and the mean value taken. Serum CRP concentrations were measured using particle-enhanced immunonephelometry (Behring BN II nephelometer, Milton Keynes, UK). Plasma cotinine concentrations were determined using high performance liquid chromatography (ABS Laboratories Ltd, London, UK) [18]. Hematocrit was determined using an automated Coulter counter (Beckman-Coulter ACT.8, High Wycombe, UK). Biochemical assays were undertaken on the fasting venous serum samples by the hospital clinical laboratory facility.

Genotyping

Genomic DNA was isolated from whole blood. For the -7351 C → T (upstream enhancer), 20 099 T → C (exon 6), and 27 445 T → A (intron 10) polymorphisms genotyping was performed by allele discrimination analysis using the 5' nuclease assay. Oligonucleotide primers and fluorescently labeled probes were obtained from Applied Biosystems (Applied Biosystems, Foster City, CA, USA) on the basis of previously published DNA and primer sequences [13,15]. In brief, polymorphic target sequences were amplified by polymerase chain reaction (PCR) in the presence of two fluorogenic probes specific for each allele. Probes hybridize to their target during the annealing step and the reporter dye is cleaved by the 5' nuclease activity of *Taq* polymerase. Real time quantitative PCR was performed in

an ABI PRISM[®] 7900HT Sequence Detector System (Applied Biosystems). Thermal cycling conditions were two initial holds (50 °C for 2 min and 95 °C for 10 min) followed by a 40-cycle two-step program (95 °C for 15 s and 60 °C for 1 min) utilizing a standardized operating protocol (WTCRF Genetics Core, Edinburgh UK; <http://www.wtcrf.ed.ac.uk>; accessed 27 July 2006). The fluorescent contribution of each dye was determined directly after the completion of PCR.

For the *Alu*-intronic polymorphism, the insertion specific sequence was submitted to the Applied Biosystems Assay-by-design service. Gene expression assays consisted of unlabeled PCR primers and TaqMan[®] MGB probes (FAM dye labeled). Forward and reverse primers were 5'-CGTAACAGGACAG-CTCACAGTT-3 and 5'-CCCTAGGAGAACTTCTCTTTA-ACTTTGTT-3, respectively. The insertion specific probe was labeled in its 5' end with FAM and in its 3' end with a NFQ (non fluorescent quencher): FAM-ACCCTATGAGATTAGAACAC-NFQ. Real time quantitative PCR was performed with a ABI PRISM[®] 7900HT Sequence Detector using the standardized operating protocol described (WTCRF Genetics Core; <http://www.wtcrf.ed.ac.uk>; accessed 27 July 2006). *Alu*-insertion PCR products were normalized to threshold values against an endogenous control (single copy RNase P gene). Each sample was run in triplicate with both the gene of interest and the RNase P control. Genotype for the *Alu*-intronic polymorphism was calculated from differences in the threshold cycle between the RNase P and *Alu*-insertion sequence.

A confirmatory assay of *Alu* Ins/Del genotypes utilized PCR amplification of the Ins fragment with primers designed to flank the region of interest (PLAT.1 5' to 3' GTGAAAAGC-AAGGTCTACCAG and PLAT.2 5' to 3' GACACCGAG-TTCATCTTGAC). PCR was performed on a DNA engine tetrad from MJ Research (Bio-Rad Laboratories, Inc, Hercules, CA, USA). Thermal cycling conditions were 94 °C for 2 min followed by 35 cycles (30 s at 94 °C for and 30 s at 60 °C and 30 s at 72 °C) with a final extension step of 10 min at 72 °C. A 10-μL sample of this reaction was analyzed on a 2.5% agarose gel and following electrophoresis DNA was visualized with ethidium bromide and UV transillumination. Amplification of the products produces a 570 bp fragment from chromosomes with the Ins allele and a 260-bp fragment from those without (Del). All genotyping was performed by an individual without information on case status or vascular study data.

Drugs

Substance P (2, 4 and 8 pmol min⁻¹; Clinalfa AG, Switzerland) and sodium nitroprusside (2, 4 and 8 μg min⁻¹; David Bull Laboratories, Warwick, UK) were administered following dissolution in 0.9% saline. All solutions were freshly prepared on the day of study.

Forearm study protocol

Subjects were requested to abstain from alcohol for 24 h, and food, caffeine-containing drinks and tobacco for at least

4 h before each study. Cardioactive medications were withheld on the morning of each study visit. All studies were conducted in a quiet temperature controlled room maintained at 22–25 °C. Patients rested recumbent, strain gauges and cuffs were applied. A 17-G venous cannula was inserted into the antecubital vein of each arm and the brachial artery of the non-dominant arm was cannulated with a 27-SWG needle (Cooper's Needle Works Ltd, Birmingham, UK).

Forearm blood flow was measured in both forearms by venous occlusion plethysmography as previously described [5,20]. Following a 30-min saline infusion to allow equilibration and recording of resting blood flow, intra-arterial substance P at 2, 4 and 8 pmol min⁻¹ (endothelium-dependent vasodilator releasing t-PA) and sodium nitroprusside at 2, 4 and 8 µg min⁻¹ (endothelium-independent vasodilator which does not release t-PA) [20] were each administered for 6 min in a randomized order with a 15 min saline washout period between each agent. Forearm blood flow was measured at baseline and with each dose of vasodilator. Venous blood sampling for determining plasma t-PA and PAI-1 antigen concentrations was undertaken at baseline and following the recording of blood flow response to each dose of substance P. Blood pressure and heart rate were measured throughout each study using a semi-automated non-invasive sphygmomanometer (Omron 705 IT; Omron Healthcare UK, Milton Keynes, UK).

Statistical analysis

Plethysmographic data were extracted from Chart data files and forearm blood flow calculated for individual venous-occlusion cuff inflations by use of a template spreadsheet (Excel 2002®; Microsoft™, Seattle, WA, USA). The last five flow recordings in each 3-min measurement period were calculated and averaged for each arm. Estimated net release of t-PA antigen was defined [5,20] as the product of the infused forearm plasma flow (based on the hematocrit and the infused forearm blood flow), and the concentration difference between the infused and non-infused forearms. Area under the curves (AUCs) for the dose–response of t-PA release were calculated using the trapezoid rule.

Allele frequencies were estimated by gene counting. Chi-squared test was used to evaluate deviations of genotype distributions from the Hardy–Weinberg equilibrium. The LD coefficient D' was calculated as previously described [21]. Baseline characteristics of the study population are presented as frequencies or mean values and standard error of mean. Differences between groups were compared by Student's *t*-test, chi-squared test, and, where appropriate, by analysis of variance (ANOVA) with repeated measures. As CRP values were not normally distributed they were log transformed prior to analysis. All statistical calculations were undertaken using GRAPHPAD PRISM (GraphPad Software, San Diego, CA, USA). Statistical significance was assigned at the 5% level.

Table 1 Allele frequencies and pairwise linkage disequilibrium (LD) coefficient between polymorphisms at the tissue plasminogen activator (t-PA) locus; (chi-squared, degrees of freedom), *P* < 0.01 for all

		Allelic frequency	Alu	-7351	20 099	27 445
Alu	Ins → Del	0.65/0.35		0.63 (67.4; 4)	0.83 (107.8; 4)	0.60 (76.7; 4)
-7351	C → T	0.69/0.31			0.85 (93.7; 4)	0.68 (82.3; 4)
20 099	T → C	0.63/0.37				0.66 (98.5; 4)
27 445	T → A	0.73/0.27				

Results

A total of 96 patients were recruited with a cardiovascular risk factor profile in keeping with their diagnosis of CHD and most were prescribed secondary preventative medications. The genotype distributions for the *Alu* insertion/deletion (II 46%, ID 36% and DD 18%), -7351 C/T enhancer (CC 54%, CT 32%, and TT 14%), 20 099 T/C exon 6 (TT 42%, CT 43%, and CC 15%) and 27 445 T/A intron 10 (TT 56%, TA 34% and AA 10%) were in keeping with the Hardy–Weinberg equilibrium. There was significant LD between the four polymorphisms (Table 1) with allelic frequencies being similar to published studies of European Caucasian subjects [13,17]. There was complete agreement between the two PCR methods in the determination of *Alu* insertion/deletion genotype. Three subjects could be genotyped only for the *Alu* insertion/deletion variant.

Biometric variables were similar between genotypes (Tables 2 and 3; data on file, University of Edinburgh, Edinburgh, UK) although there were significantly fewer smokers in the -7351 heterozygote group (*P* = 0.01, chi-squared).

Hemodynamics and forearm blood flow

Resting heart rate, blood pressure and non-infused forearm blood flow were similar in each group and these did not change significantly during infusion of either vasodilator (Tables 2–4; data on file).

Substance P and sodium nitroprusside increased the infused forearm blood flow in all subjects (*P* < 0.001, ANOVA). None of the genetic polymorphisms appeared to influence the endothelium-dependent or -independent blood flow responses (Fig. 1; data on file).

Plasma fibrinolytic variables

Baseline plasma t PA and PAI 1 antigen concentrations were similar for each genotype (Table 4; data on file). Substance P caused a dose-dependent increase in plasma t-PA antigen within the infused arm in all three groups (*P* < 0.01, ANOVA) whilst plasma PAI-1 antigen concentrations were unchanged (data on file). There were no differences in substance P-induced t-PA release between any of the genetic polymorphisms (Fig. 2; data on file). Similarly, the AUC for net t-PA release over the

Table 2 Clinical characteristics of subjects according to *Alu* insertion/deletion genotype (*n* = 96)

	Genotype			<i>P</i> [†]
	II (<i>n</i> = 44)	ID (<i>n</i> = 35)	DD (<i>n</i> = 17)	
Age, years	59 ± 1	60 ± 1	59 ± 2	0.88
Gender, male/female	33/11	32/3	14/3	0.16
BMI, kg m ⁻²	29 ± 1	28 ± 1	30 ± 1	0.27
Systolic BP, mmHg	136 ± 3	128 ± 4	132 ± 4	0.23
Diastolic BP, mmHg	79 ± 2	75 ± 2	73 ± 2	0.30
Pulse, bpm	57 ± 1	58 ± 2	57 ± 3	0.96
Current smoker (%)	30	23	47	0.42
Co-morbidity (%)				
Previous MI	43	46	24	0.28
Hypertension	50	49	47	0.98
FHx CHD	52	37	29	0.74
Prior hyperlipidemia	93	94	94	1.00
Extent of coronary disease, (%)*				
1 vessel	41	51	41	0.33
2 vessels	27	20	24	0.86
3 vessels	30	17	35	0.43
Medical therapy, (%)				
Aspirin	100	100	94	0.10
Statin therapy	91	97	94	0.52
ACE inhibitor, ARB	27	34	29	1.00
Urea, mmol L ⁻¹	5.4 ± 0.2	5.6 ± 0.3	5.7 ± 0.4	0.70
Creatinine, µmol L ⁻¹	93.1 ± 2.5	94.6 ± 2.1	91.3 ± 2.6	0.72
Glucose, mmol L ⁻¹	5.6 ± 0.1	5.7 ± 0.2	6.0 ± 0.2	0.24
Total chol, mmol L ⁻¹	4.5 ± 0.2	4.4 ± 0.2	4.4 ± 0.2	0.89
LDL chol, mmol L ⁻¹	2.6 ± 0.1	2.5 ± 0.2	2.2 ± 0.2	0.28
Triglycerides, mmol L ⁻¹	1.7 ± 0.2	1.7 ± 0.2	2.3 ± 0.3	0.09
CRP, mg L ⁻¹	1.87 ± 0.18	1.92 ± 0.35	1.90 ± 0.41	0.97

Values are mean ± SEM.

*Angiographic data unavailable on five subjects. †*P* = ANOVA or chi-squared for difference between genotypes. BMI, body mass index; bpm, beats per minute; CHD, coronary heart disease; MI, myocardial infarction; FHx, family history; chol, cholesterol; ACE, angiotensin converting enzyme; ARB, angiotensin receptor blocker; CRP, C-reactive protein.

30-min period of substance P infusion was similar between genotypes (data on file).

Smoking status was confirmed by plasma cotinine concentration (median 307.8 vs. 0.6 ng mL⁻¹ in smokers and non-smokers respectively). Subgroup analysis revealed an interaction between smoking status and vascular function. Consistent with our previous findings, cigarette smokers had impaired forearm blood flow (FBF) responses (*P* < 0.001, ANOVA) and net t-PA release (*P* = 0.05, ANOVA; Fig. 3) compared to non-smokers. In light of the uneven distribution of smokers across -7351 genotypes and the interaction between smoking and t-PA release, we performed a *post hoc* analysis of t-PA release according to smoking status. However, we found no difference in the AUC for net t-PA release between CC, CT and TT -7351 genotypes in either smokers (111 [48–174]; 167 [94–240]; 193 [5–382]; *P* = 0.41 ANOVA) or non-smokers (187 [114–260]; 160 [100–220]; 188 [50–325]; *P* = 0.83) respectively (mean [95% confidence intervals]).

Table 3 Clinical characteristics of subjects according to -7351 C/T genotype (*n* = 93)

	Genotype			<i>P</i> [†]
	CC (<i>n</i> = 50)	CT (<i>n</i> = 30)	TT (<i>n</i> = 13)	
Age, years	59 ± 1	59 ± 1	61 ± 3	0.82
Gender, male/female	38/12	27/3	11/2	0.28
BMI, kg m ⁻²	29 ± 1	28 ± 1	29 ± 1	0.47
Systolic BP, mmHg	134 ± 3	131 ± 4	123 ± 6	0.27
Diastolic BP, mmHg	79 ± 2	75 ± 2	70 ± 3	0.07
Pulse, bpm	57 ± 1	57 ± 2	54 ± 3	0.57
Current smoker (%)	46	13	38	0.01
Co-morbidity (%)				
Previous MI	44	43	38	0.89
Hypertension	46	40	62	0.43
FHx CHD	32	37	31	0.23
Prior hyperlipidemia	92	93	92	0.98
Extent of coronary disease, (%)*				
1 vessel	40	50	38	0.64
2 vessels	24	23	23	0.99
3 vessels	30	20	38	0.42
Medical therapy, (%)				
Aspirin	100	97	100	0.35
Statin therapy	88	100	92	0.14
ACE inhibitor, ARB	28	23	54	0.98
Urea, mmol L ⁻¹	5.2 ± 0.2	5.7 ± 0.2	6.1 ± 0.5	0.09
Creatinine, µmol L ⁻¹	93.3 ± 2.2	92.6 ± 2.1	92.3 ± 4.0	0.96
Glucose, mmol L ⁻¹	5.6 ± 0.1	5.6 ± 0.2	6.0 ± 0.3	0.26
Total chol, mmol L ⁻¹	4.6 ± 0.2	4.4 ± 0.2	4.2 ± 0.3	0.37
LDL chol, mmol L ⁻¹	2.7 ± 0.1	2.6 ± 0.1	2.0 ± 0.3	0.08
Triglycerides, mmol L ⁻¹	1.8 ± 0.2	1.6 ± 0.1	2.4 ± 0.3	0.08
CRP, mg L ⁻¹	2.02 ± 0.29	1.5 ± 0.3	2.3 ± 0.7	0.49

Values are mean ± SEM.

*Angiographic data unavailable on five subjects. †*P* = ANOVA or chi-squared for difference between genotypes. BMI, body mass index; bpm, beats per minute; CHD, coronary heart disease; MI, myocardial infarction; FHx, family history; chol, cholesterol; ACE, angiotensin converting enzyme; ARB, angiotensin receptor blocker; CRP, C-reactive protein.

Discussion

This is the first study to assess directly the role of *t-PA* genetic polymorphisms in determining acute t-PA release in patients with CHD. Using a validated and reproducible technique [22], we have found no difference in the basal concentration, or net release of, plasma t-PA antigen between genotypes. These important findings suggest that, in patients with established coronary atherosclerosis, variation at the *t-PA* gene locus does not substantially influence acute endogenous fibrinolytic capacity.

Tissue plasminogen activator release from endothelial cells is the major arbiter of local intravascular fibrinolysis [23] and will influence vessel patency following acute coronary thrombosis [24]. Inter-individual differences in t-PA release may contribute to a pro-thrombotic phenotype and determine the sequelae of acute MI. Indeed, clustering of individuals with low rates of t-PA release has been reported in families with a history of thrombosis [25]. Moreover, genetic murine models of t-PA

Table 4 Effect of *Alu* insertion/deletion and -7351 C/T genotypes on baseline forearm blood flow, tissue type-plasminogen activator (t-PA) and plasminogen activator inhibitor type 1 (PAI-1) antigen concentrations; *P* = ns between genotypes

	<i>Alu</i> Insertion/deletion			-7351 C/T		
	II	ID	DD	CC	CT	TT
Forearm blood flow (mL 100 mL tissue ⁻¹ min ⁻¹)						
Infused arm	2.7 ± 0.1	2.8 ± 0.2	2.7 ± 0.2	2.6 ± 0.1	2.8 ± 0.2	2.6 ± 0.3
Non-infused arm	2.7 ± 0.2	2.7 ± 0.2	2.5 ± 0.2	2.6 ± 0.1	2.9 ± 0.2	2.4 ± 0.3
t-PA antigen (ng mL ⁻¹)						
Infused arm	9.1 ± 0.5	10.7 ± 0.8	9.0 ± 0.7	9.4 ± 0.5	9.8 ± 0.8	9.3 ± 1.1
Non-infused arm	9.3 ± 0.5	10.9 ± 0.8	8.8 ± 0.7	9.6 ± 0.5	9.9 ± 0.8	9.3 ± 1.1
PAI-1 antigen (ng mL ⁻¹)						
Infused arm	43.3 ± 4.2	43.6 ± 3.7	44.1 ± 6.6	44.5 ± 3.5	44.3 ± 4.8	39.8 ± 7.4
Non-infused arm	46.0 ± 4.2	44.0 ± 3.6	43.7 ± 6.5	47.7 ± 3.6	42.4 ± 4.6	40.7 ± 7.2

Values are mean ± SEM.

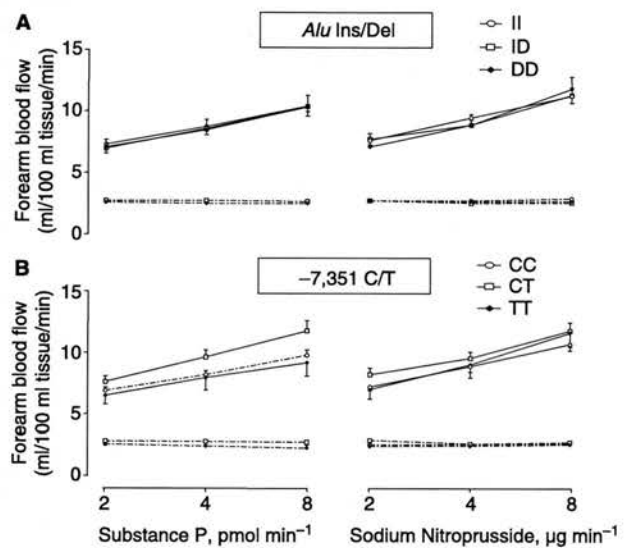


Fig. 1. Infused (solid line) and non-infused (dashed line) forearm blood flow during intra-brachial substance P (left) and sodium nitroprusside (right) according to (A) *Alu* insertion/deletion, and (B) -7351 C/T, genotypes. *P* < 0.001, ANOVA, for infused FBF response to both vasodilators.

deficiency show a reduced thrombolytic potential and an increased thrombotic tendency [26]. We therefore wanted to explore the potential genetic influences on t-PA release in at risk patients with CHD.

The t-PA -7351C → T enhancer site polymorphism has been associated with an increased risk of first MI [15] and lacunar stroke [16] although the data on non-lacunar stroke is conflicting [16,27]. The strong linkage between the -7351 C/T enhancer and the *Alu*-repeat polymorphisms may explain the association between the (non-coding) *Alu* insertion/deletion polymorphism and cardiovascular events [12,13]. In healthy volunteers (*n* = 51), both the *Alu* insertion/deletion and -7351 C → T enhancer polymorphisms were associated with significant differences in basal t-PA release with II and CC homozygotes having around 2-fold greater release rates than the DD and TT homozygotes respectively [13,17]. Of the genotypes studied here, the 7351 C → T substitution is thought to result in a functional change occurring within an Sp1 transcription

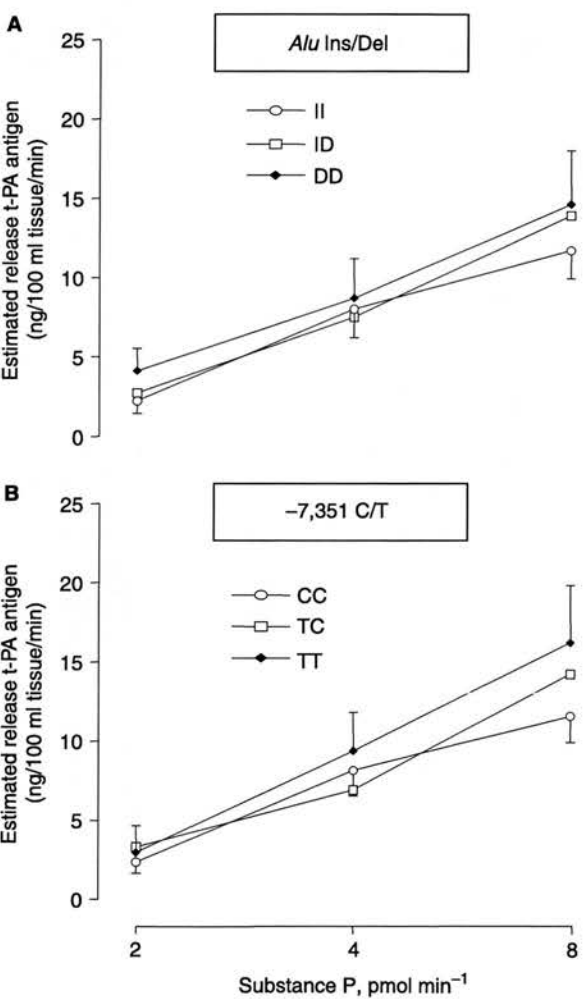


Fig. 2. Estimated net release of tissue plasminogen activator (t-PA) antigen induced by intra-brachial substance P (2, 4 and 8 pmol min⁻¹) according to (A) *Alu* insertion/deletion, and (B) -7351 C/T, genotypes. *P* < 0.001, ANOVA, for dose response.

factor binding site which is negatively regulated by binding of the thymidine allele [13]. However despite this difference in basal t-PA release rate, acute t-PA release was similar between all genotypes. Now, in a larger cohort of subjects (*n* = 96) with

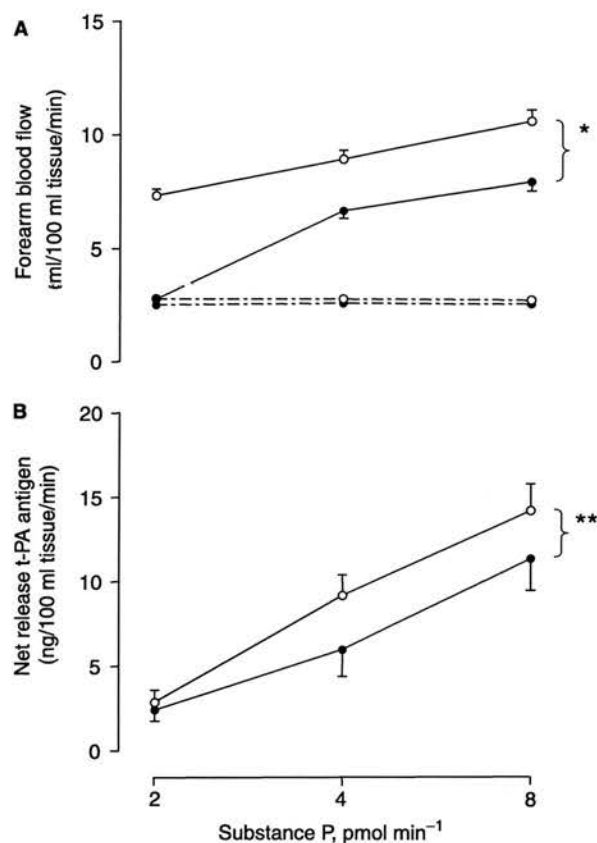


Fig. 3. Infused (solid line) and non-infused (dashed line) forearm blood flow (A), and net t-PA release (B), in response to intra-brachial substance P in smokers (filled circle) and non-smokers (hollow circle). * $P < 0.001$, 2-way ANOVA, infused FBF response in smokers vs. non-smokers; ** $P = 0.05$, 2-way ANOVA, net t-PA release in smokers vs. non-smokers.

established CHD, we have shown that there is no significant relationship between basal plasma t-PA concentrations or acute t-PA release, and these genetic polymorphisms. Furthermore, endothelium-dependent vasodilatation, a complementary measure of endothelial function, was similarly unaffected by variation within the *t-PA* gene locus.

Regulatory control of t-PA release involves distinct pathways from those controlling vessel calibre and indeed t-PA release can be induced without changes in forearm blood flow [28]. In view of previous work in healthy volunteers [13,17], we chose to assess whether a number of *t-PA* genetic polymorphisms were associated with changes in vascular function in patients with CHD. If t-PA genotype was important in regulating endogenous t-PA release, certain alleles could favor the continued presence of thrombus following atheromatous plaque rupture. Organization of residual thrombus is known to stimulate further plaque growth and expansion [29], disrupting endothelial integrity that is critical to the regulation of vascular tone and coagulation. Impaired endothelium-dependent vasodilatation is considered a surrogate of endothelial dysfunction and has been widely studied as a bio-marker of atherosclerosis. We therefore hypothesized that *t-PA* genetic polymorphisms might also be associated with changes in endothelium-depend-

ent vasodilatation through pathways linking coagulation, atherogenesis, and endothelial dysfunction. However, we found no evidence to support the contention that t-PA polymorphisms influence endothelial function in patients with CHD. In contrast, cigarette smoking and CHD are associated with endothelial dysfunction as demonstrated by impaired endothelium-dependent vasodilatation [1] and endogenous t-PA release [5,30]. Our study confirms these previous findings in cigarette smokers in a large cohort of patients with established CHD maintained on secondary preventative medications. These consistent findings would appear to reaffirm the validity of our approach and suggest that environmental rather than genetic factors have a more dominant influence on acute endogenous t-PA release.

There are a number of differences between the current study and previous reports demonstrating an interaction between acute t-PA release and polymorphisms within the *t-PA* gene locus. Our population comprised subjects with established CHD recruited within the UK. In contrast, Jern *et al.* [13,17] studied healthy Scandinavian volunteers free of symptomatic atherosclerosis. A recent meta-analysis of studies on genetic polymorphisms and CHD risk demonstrated heterogeneity according to the population studied [31]. The *Alu*-repeat polymorphism was associated with an increased incidence of acute MI in Dutch subjects [12] but not in studies from North America [11] or the UK [32]. Our subjects were all of European Caucasian origin, recruited from within the same geographical area, and had similar risk factor profiles. It seems unlikely that biological variability or modulation from environmental factors obscured true differences in t-PA release between genotypes. Although our findings may not be applicable to other racial groups, Rosenbaum and colleagues [33] failed to demonstrate differences in acute endogenous t-PA release between healthy black and white Americans.

Study limitations

Although endothelium-dependent blood flow responses are similar in the forearm and coronary circulations [34], we cannot be sure that these polymorphisms of the *t-PA* gene do not influence fibrinolytic responses within the coronary circulation. Indeed, there are limited data to suggest that, although not apparent in the forearm circulation, angiotensin-converting enzyme gene insertion/deletion polymorphism can influence acute t-PA release in the coronary circulation [35].

Jern and colleagues previously demonstrated a difference in basal, though not stimulated, t-PA release according to t-PA genotype [13,17]. Whilst we cannot determine basal t-PA release using the venovenous technique described, we have found no relationship between t-PA genotype and stimulated t-PA release. Calculation of net t-PA release provides an accurate assessment of stimulated t-PA release with good reproducibility [19], and basal release contributes only a small proportion of the overall venous plasma t-PA

concentration. In this study we did not find any difference in acute t-PA release in response to substance P between t-PA genotypes. However, our study protocol cannot exclude the possibility that these polymorphisms might alter protein transcription and endothelial storage, or the very early (< 6 min) release of, t-PA in response to substance P infusion [36].

In our cohort, there was an uneven distribution of smokers between t-PA -7351 CC, CT and TT groups. Although we found no difference in net t-PA release between these three groups when analyzed according to smoking status, we accept that the relationship between cigarette smoking and impaired t-PA release may have obscured an association between t-PA genotype and fibrinolytic function with the *post hoc* analysis limited by the number of smokers and non-smokers within each group.

Conclusion

Whilst we have confirmed previous findings showing that cigarette smokers have impaired endothelial vasomotor and fibrinolytic function, we have found no major effect of genetic variation within the t-PA gene locus on either plasma t-PA concentrations or acute t-PA release in subjects with stable CHD.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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Vascular and fibrinolytic effects of intra-arterial tumour necrosis factor- α in patients with coronary heart disease

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A B S T R A C T

Elevated plasma t-PA (tissue plasminogen activator) and serum CRP (C-reactive protein) concentrations are associated with an adverse cardiovascular risk. In the present study, we investigated whether acute local inflammation causes vascular dysfunction and influences t-PA release in patients with stable coronary heart disease. Serum CRP, plasma t-PA and PAI-1 (plasminogen activator inhibitor type 1) concentrations were determined in 95 patients with stable coronary heart disease. A representative subpopulation of 12 male patients received an intra-brachial infusion of TNF- α (tumour necrosis factor- α) and saline placebo using a randomized double-blind cross-over study design. Forearm blood flow and plasma fibrinolytic and inflammatory variables were measured. Serum CRP concentrations correlated with plasma t-PA concentrations ($r = 0.37$, $P < 0.001$) and t-PA/PAI-1 ratio ($r = -0.21$, $P < 0.05$). Intra-arterial TNF- α caused a rise in t-PA concentrations ($P < 0.001$) without affecting blood flow or PAI-1 concentrations. TNF- α pretreatment impaired acetylcholine- and sodium nitroprusside-induced vasodilatation ($P < 0.001$ for both) whilst doubling bradykinin-induced t-PA release ($P = 0.006$). In patients with stable coronary heart disease, plasma fibrinolytic factors correlate with a systemic inflammatory marker and local vascular inflammation directly impairs vasomotor function whilst enhancing endothelial t-PA release. We suggest that the adverse prognosis associated with elevated plasma t-PA concentrations relates to the underlying causative association with vascular inflammation and injury.

INTRODUCTION

In epidemiologic studies of patients with CHD (coronary heart disease) [1] and in prospective studies in healthy populations [2], higher plasma concentrations of the pro-fibrinolytic factor t-PA (tissue plasminogen activator)

positively and independently predict future cardiovascular events. It would be anticipated that high t-PA concentrations would protect against subsequent cardiovascular events rather than the reverse. This paradoxical association is, in part, explained by the concomitant elevation of PAI-1 (plasminogen activator inhibitor

Key words: coronary disease, endothelial function, fibrinolysis, inflammation, tumour necrosis factor- α (TNF- α), vasodilation.

Abbreviations: BP, blood pressure; CHD, coronary heart disease; CRP, C-reactive protein; F1 + 2, fragment 1 and 2; FBF, forearm blood flow; HR, heart rate; hs-CRP, highly sensitive CRP; IL-6, interleukin-6; IU, international units; NO, nitric oxide; eNOS, endothelial NO synthase; ns, not significant; PAI-1, plasminogen activator inhibitor type 1; SBP, systolic blood pressure; SNP, sodium nitroprusside; TNF- α , tumour necrosis factor- α ; t-PA, tissue plasminogen activator.

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type 1) which complexes with, and inactivates, t-PA. However, the precise stimulus for this increased t-PA release remains unclear.

Areas of endothelial denudation and thrombus deposition are a common finding on the surface of atherosclerotic plaques and are usually subclinical. Through t-PA release, endogenous fibrinolysis is usually able to prevent thrombus propagation, although organization of the residual thrombus may lead to plaque growth and expansion [3]. The adverse prognosis conferred by elevated plasma t-PA antigen concentrations may, therefore, reflect the extent of occult atheroma and subclinical plaque rupture stimulating t-PA release.

Markers of systemic inflammation, such as CRP (C-reactive protein) and TNF- α (tumour necrosis factor- α), are elevated in patients with cardiovascular disease [4,5]. Indeed, serum CRP concentrations predict the development of cardiovascular disease independently of other risk factors. Previous studies have indicated a direct relationship between serum CRP and plasma t-PA concentrations [6,7]. This raises the question of whether vascular inflammation is causally related to the elevation in plasma t-PA concentrations or whether CRP and t-PA are independently increased by a common factor related to the atherosclerotic process itself, such as acute plaque rupture.

Abnormalities of endothelial function have been demonstrated in patients with atherosclerosis [8] and vascular inflammation [9,10]. In patients with CHD, restoration of endothelium-dependent vasomotor function occurs when there is normalization of CRP concentrations [11], whereas ongoing chronic inflammation is associated with an impaired fibrinolytic response to venous occlusion [12]. As the endothelium is the major source of plasma t-PA, abnormalities of endothelial function may therefore mediate the potential inflammation-induced elevations in plasma t-PA concentrations.

It therefore remains unclear whether elevated t-PA concentrations are implicated in the mechanisms contributing to, or arise as a consequence of, atherothrombotic events.

The aims of the present study were, in patients with stable CHD, to confirm the previous association between plasma CRP and t-PA concentrations and to determine the effect of acute local vascular inflammation provoked by direct intra-arterial infusion of TNF- α on vasomotor function and endothelial t-PA release.

METHODS

Patients

We recruited patients with CHD confirmed by angiography (defined as > 70% luminal stenosis of at least one major epicardial coronary vessel) or a previous history of Q-wave myocardial infarction. All patients had stable

anginal symptoms and had not undergone coronary revascularization within the preceding 3 months. Exclusion criteria were significant cardiac failure, renal impairment, SBP [systolic BP (blood pressure)] < 100 or > 190 mmHg, diabetes mellitus, history or clinical features of recent infective illness and immunosuppressive or non-steroidal anti-inflammatory medication (excluding 75 mg/day aspirin). All studies were undertaken with the approval of the local Research Ethics Committee and in accordance with the Declaration of Helsinki. Written informed consent was obtained from each subject.

Venous sampling and assays

Plasma t-PA, PAI-1 (Coalizer[®]; Chromogenix), prothrombin F1 + 2 (fragment 1 and 2; Enzygnost F1 + 2; Dade Behring), TNF- α (Quantikine; R&D Systems) and IL-6 (interleukin-6; Dako) concentrations were determined using ELISAs, and t-PA activity using a photometric method (Coatest t-PA; Chromogenix) [13–15]. Assays of hs-CRP (highly sensitive CRP) were undertaken using the method of particle-enhanced immunonephelometry (BN II nephelometer; Behring). Venous blood was collected into tubes containing acidified buffered citrate (for t-PA), trisodium citrate (for PAI-1 and prothrombin F1 + 2), potassium EDTA (for cytokines) and serum gel tubes (for CRP). Platelet-free plasma and serum were stored at -80°C before assay. Haematocrit and white cell count were determined using an automated Coulter counter. Biochemical assays were undertaken on fasting venous samples by the hospital Clinical Laboratory facility.

Drugs

TNF- α (Knoll Pharmaceuticals), bradykinin (Clinalfa), acetylcholine (Novartis) and SNP (sodium nitroprusside; David Bull Laboratories) were administered following dissolution in 0.9% saline.

Study design

All subjects abstained from alcohol for 24 h and from food, tobacco and caffeine-containing drinks for at least 4 h before each study visit. A venous blood sample was taken from all patients for estimation of serum CRP and plasma t-PA and PAI-1 antigen concentrations. Twelve representative male patients were then recruited into a randomized double-blind placebo-controlled cross-over study comparing the effect of direct intra-brachial infusion of TNF- α and saline placebo.

Forearm study protocol

All studies were carried out at 09:00 hours in a quiet temperature-controlled room maintained at $22\text{--}25^{\circ}\text{C}$. Patients rested recumbent and strain gauges and cuffs were applied. A 17-gauge venous cannula was inserted into the antecubital vein of each arm, and the brachial artery of the non dominant arm was cannulated with a

27-SWG (standard wire gauge) needle (Cooper's Needle Works). FBF (forearm blood flow) was measured in both forearms by venous occlusion plethysmography using mercury in silastic strain gauges as described previously [16]. BP and HR (heart rate) were measured using a semi-automated non-invasive sphygmomanometer (Omron 705 IT).

Subjects ($n=12$) attended on two occasions at least two weeks apart and received an intra-arterial infusion of either TNF- α (80 ng/min) or saline placebo over 60 min, followed on each occasion by a further 60 min of saline infusion. Thereafter intra-arterial bradykinin (100, 300 and 1000 pmol/min), acetylcholine (5, 10 and 20 μ g/min) and SNP (2, 4 and 8 μ g/min) were administered at 1 ml/min with a 15 min saline washout period between each agent. The dose of TNF- α was chosen to achieve local cytokine concentrations comparable with healthy volunteer studies [13] and those seen in cardiovascular disease [17].

Venous blood samples for t-PA antigen and activity and PAI-1 antigen were obtained at baseline, after 60 min of TNF- α /placebo infusion, following the 60 min infusion of saline, before and during each dose of bradykinin and 15 min after the end of bradykinin infusion. Plasma cytokines, hs-CRP and prothrombin F1 + 2 were assessed prior to and following the TNF- α /placebo and saline infusions and at the end of each study.

Statistical analysis

Estimated net release of t-PA was defined previously as the product of the infused forearm plasma flow and the concentration difference between the infused and non-infused forearms [16]. Because basal t-PA concentrations were altered by pretreatment with TNF- α , net release of t-PA during bradykinin infusion was calculated by subtracting the mean t-PA release before and 15 min after cessation of bradykinin infusion [13]. The area under the curve was calculated for the estimated net release of t-PA in response to bradykinin. Data were examined, where appropriate, by ANOVA with repeated measures, followed by post-hoc Student's t tests adjusted with a Bonferroni correction for multiple comparisons. Spearman's correlation was used to compare CRP and plasma levels of fibrinolytic factors. As serum CRP concentrations have a skewed distribution, they were logarithmically transformed.

All statistical calculations were undertaken using GraphPad Prism (GraphPad Software). Results are expressed as means \pm S.E.M., unless otherwise stated, and statistical significance was assigned at the 5% level.

RESULTS

Patients had a typical cardiovascular risk factor profile in keeping with their diagnosis of CHD and most were

Table 1 Baseline characteristics of the 95 subjects with stable CHD

Values are means \pm S.D. or n (%). ACE, angiotensin-converting enzyme; ARB, angiotensin II receptor blocker; LDL, low-density lipoprotein. *Angiographic data were unavailable on one subject.

Characteristics	
Age (years)	59 \pm 7
Body mass index (kg/m ²)	29 \pm 5
Male gender (n)	79 (83 %)
Previous myocardial infarction (n)	39 (41 %)
Extent of coronary artery disease (n)*	
One vessel	42 (44 %)
Two vessels	24 (25 %)
Three vessels	28 (29 %)
Previous coronary revascularization (n)	64 (67 %)
Co-morbidity (n)	
Hypertension	46 (48 %)
Previous hyperlipidaemia	89 (94 %)
Family history of premature CHD	32 (34 %)
Smoker/ex-smoker/non-smoker	30/27/38 (32 %/28 %/40 %)
Medical therapy (n)	
Aspirin	95 (100 %)
Anti-anginal	82 (86 %)
Statin	90 (95 %)
ACE-inhibitor/ARB	30 (32 %)
Serum urea (mmol/l)	5.6 \pm 1.3
Serum creatinine (μ mol/l)	93 \pm 13
Plasma glucose (mmol/l)	5.7 \pm 0.9
Total cholesterol (mmol/l)	4.5 \pm 0.9
LDL-cholesterol (mmol/l)	2.5 \pm 0.7
Triacylglycerols (mmol/l)	1.8 \pm 0.9

prescribed secondary preventative medications (Tables 1 and 2). The majority of the subjects were male, two-thirds had previously undergone coronary revascularization and approx. one-third were habitual smokers.

Plasma fibrinolytic factors and correlation with CRP

In the cohort of 95 patients with stable CHD, serum hs-CRP concentrations correlated with plasma t-PA antigen concentrations ($r=0.37$, $P<0.001$) and plasma PAI-1 antigen concentrations ($r=0.28$, $P=0.006$) and inversely with the ratio of t-PA/PAI-1 antigen ($r=-0.21$, $P<0.05$). As anticipated, plasma t-PA antigen concentrations correlated with plasma PAI-1 antigen concentrations ($r=0.49$, $P<0.001$).

Effect of acute inflammation on vascular and fibrinolytic function

Those subjects who received TNF- α and saline placebo had similar baseline characteristics to the main cohort and all were receiving statin and aspirin therapy. There were

Table 2 Baseline characteristics of 12 patients receiving TNF- α and saline placebo in a randomized double-blind cross-over study design

Values are means \pm S.E.M or *n* (%). ACE, angiotensin-converting enzyme; LDL, low-density lipoprotein. *Angiographic data were unavailable on one subject.

Characteristics	
Age (years)	60 \pm 2
Body mass index (kg/m ²)	24 \pm 1
Male gender (<i>n</i>)	12 (100 %)
Previous myocardial infarction (<i>n</i>)	5 (42 %)
Extent of coronary disease (<i>n</i>)*	
One vessel	5 (42 %)
Two vessels	4 (33 %)
Three vessels	2 (17 %)
Previous coronary revascularization (<i>n</i>)	9 (75 %)
Co-morbidity (<i>n</i>)	
Hypertension	3 (25 %)
Family history of premature CHD	1 (8 %)
Previous hyperlipidaemia	12 (100 %)
Smoker/ex-smoker/non-smoker	2/7/3 (17%/58%/25 %)
Medical therapy (<i>n</i>)	
Aspirin	12 (100 %)
Anti-anginal	11 (92 %)
Statin	12 (100 %)
ACE-inhibitor	1 (8 %)
Serum urea (mmol/l)	5.9 \pm 0.3
Serum creatinine (μ mol/l)	93 \pm 2
Plasma glucose (mmol/l)	5.2 \pm 0.1
Total cholesterol (mmol/l)	3.9 \pm 0.2
LDL-cholesterol (mmol/l)	2.1 \pm 0.2
Triacylglycerols (mmol/l)	1.3 \pm 0.2
Placebo visit	
HR (beats/min)	56 \pm 2
SBP (mmHg)	145 \pm 6
Diastolic BP (mmHg)	83 \pm 4
FBF (ml \cdot 100 ml ⁻¹ of tissue \cdot min ⁻¹)	
Infused arm	2.1 \pm 0.2
Non-infused arm	2.0 \pm 0.2
TNF- α visit	
HR (beats/min)	56 \pm 3
SBP (mmHg)	142 \pm 4
Diastolic BP (mmHg)	81 \pm 2
FBF (ml \cdot 100 ml ⁻¹ of tissue \cdot min ⁻¹)	
Infused arm	2.0 \pm 0.2
Non-infused arm	2.1 \pm 0.2

no differences in resting arterial pressure, HR or FBF between the two study visits (Table 2).

There was no change in HR or BP during infusion of either TNF- α or placebo. Haematocrit, temperature, white cell count and hs-CRP were similar on both study visits [*P* = ns (not significant); results not shown]. The study protocol was well tolerated with no major adverse effects.

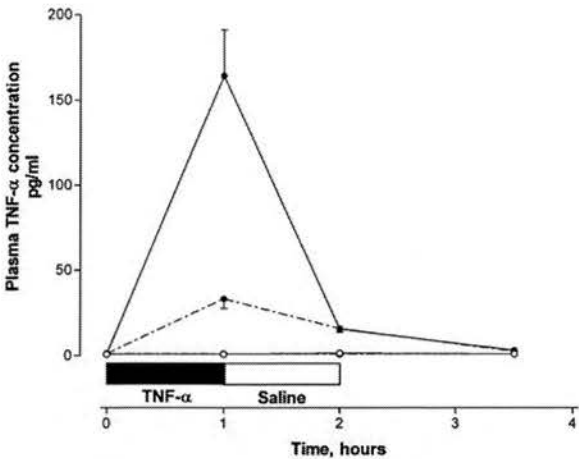


Figure 1 Plasma TNF- α concentrations in the infused (solid line) and non-infused arm (dashed/dotted line) after 60 min of intra-arterial TNF- α (●) and saline placebo (○) *P* < 0.001 for the TNF- α dose-response in the infused arm, as determined by ANOVA; *P* < 0.001 when TNF- α compared with saline placebo in the infused arm, as determined by ANOVA.

Plasma cytokine concentrations

Intra-arterial infusion of TNF- α increased plasma TNF- α concentrations from 1.4 ± 0.2 to 164.5 ± 26.8 pg/ml in the infused arm and from 1.3 ± 0.1 to 33.3 ± 5.6 pg/ml in the non-infused arm (*P* < 0.001; Figure 1). At 1 h after TNF- α infusion, plasma IL-6 concentrations increased from 4.1 ± 1.9 to 6.4 ± 2.3 pg/ml in the infused arm (*P* < 0.001, as determined by ANOVA), but were unchanged in the non-infused arm (4.0 ± 1.9 to 3.7 ± 1.3 pg/ml). Placebo infusion had no effect on plasma IL-6 concentrations in the infused arm (3.9 ± 1.7 to 4.3 ± 1.3 pg/ml; *P* = ns). Serum CRP concentrations were unchanged following the 60 min infusion of TNF- α and saline placebo.

Vasomotor and fibrinolytic responses

There was no significant change in resting FBF in the 2 h after the start of either TNF- α or placebo infusion. After 60 min of TNF- α , plasma t-PA antigen and activity concentrations in the infused arm had increased from 9.4 ± 1.1 to 11.3 ± 1.2 ng/ml and 0.3 ± 0.1 to 2.1 ± 0.6 IU (international units)/ml respectively (*P* < 0.001), and these remained elevated in the 2 h following discontinuation of the TNF- α infusion (Figure 2). There was no change in plasma PAI-1 antigen concentrations in the infused arm and no change in either plasma t-PA or PAI-1 concentrations in the non-infused arm during the study (results not shown). Prothrombin F1 + 2 concentrations in the infused arm were unaltered following either saline placebo (0.9 ± 0.1 to 0.9 ± 0.1 ng/ml; *P* = ns) or TNF- α infusion (0.9 ± 0.1 to 1.0 ± 0.1 ng/ml; *P* = ns).

There was a dose-dependent increase in FBF during bradykinin, acetylcholine and SNP infusion (*P* \leq 0.01,

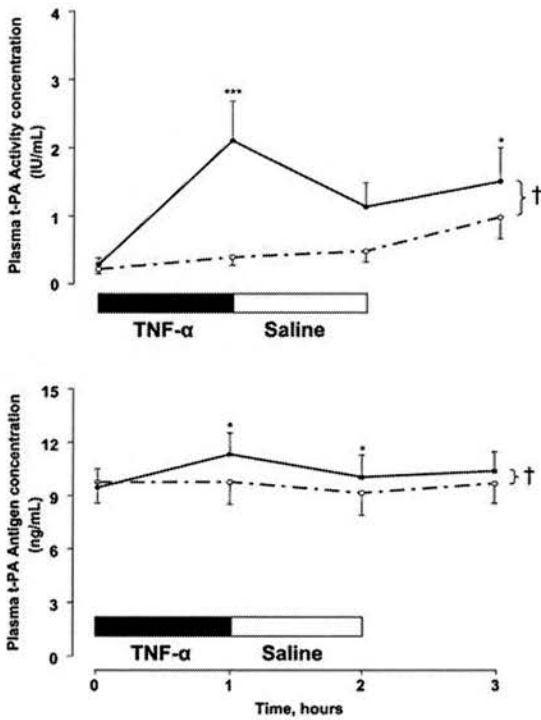


Figure 2 Plasma t-PA activity (upper panel) and antigen (lower panel) concentrations in the infused (●) and non-infused (○) arms after 60 min infusion of intra-arterial TNF- α

† $P < 0.001$ when infused compared with non-infused arms, as determined by ANOVA. * $P < 0.05$ and *** $P < 0.001$, as determined by post-hoc Student's t test for treatment effect.

as determined by ANOVA). Compared with the saline placebo, TNF- α pretreatment impaired acetylcholine- and SNP-induced vasodilatation ($P < 0.001$ for both), but did not alter the response to bradykinin (Figure 3).

Plasma t-PA concentrations increased in a dose-dependent manner during bradykinin infusion on both study

visits ($P < 0.001$). Pretreatment with TNF- α augmented the bradykinin-induced rise in plasma t-PA antigen and activity concentrations ($P < 0.001$ for both; Figure 4) and significantly increased estimated net release of t-PA antigen (63.7 ± 14.8 compared with 120.6 ± 26.1 ng \cdot 100 ml $^{-1}$ of tissue \cdot min $^{-1}$ at peak dose; $P < 0.05$) and activity (54.8 ± 14.8 compared with 98.8 ± 21.0 IU \cdot 100 ml $^{-1}$ of tissue \cdot min $^{-1}$ at peak dose; $P < 0.05$). Over the 30 min period of bradykinin infusion, TNF- α increased the area under the curve for net t-PA antigen and activity release by 120 % and 188 % respectively ($P = 0.006$).

Subgroup analysis showed a significant impairment of endothelium-dependent vasodilatation as well as bradykinin-induced t-PA response in cigarette smokers. Qualitatively the effect of intra-arterial TNF- α on the blood flow and fibrinolytic responses was similar in both smokers and non-smokers.

DISCUSSION

In the present study, we have confirmed the direct association between plasma t-PA and serum CRP concentrations in patients with stable CHD. For the first time, we have extended this observation using an acute local vascular inflammatory model and demonstrated that direct intra-arterial infusion of TNF- α causes a slow onset and sustained increase in basal t-PA release. This arterial inflammation was also associated with increased stimulated t-PA release in the presence of impaired vasomotor function. Our findings are consistent with the suggestion that t-PA is released during vascular inflammation and endothelial injury and this may, in part, explain the adverse prognosis associated with increased plasma t-PA concentrations.

The link between markers of inflammation and plasma t-PA concentrations suggests that vascular inflammation and injury may be responsible for endothelial t-PA

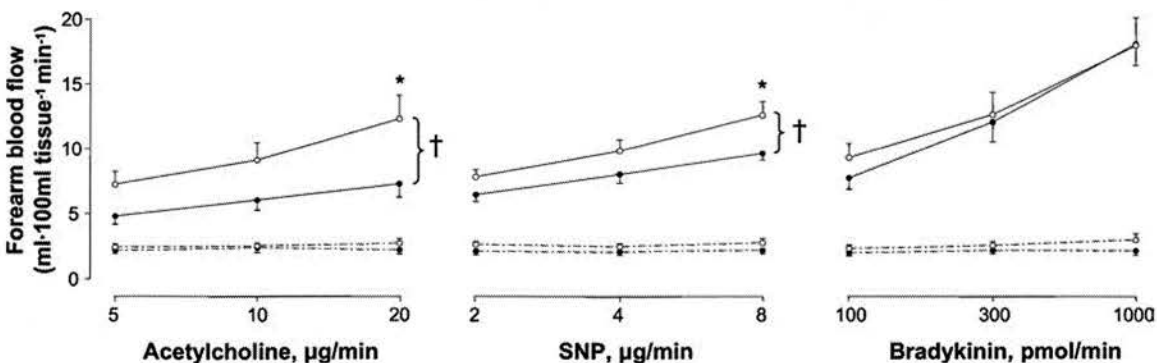


Figure 3 Infused (solid line) and non-infused (dashed/dotted line) FBF during incremental doses of acetylcholine (left-hand panel), SNP (middle panel) and bradykinin (right-hand panel) following pretreatment with TNF- α (●) or saline placebo (○)

$P \leq 0.01$ for all infused arm responses, as determined by ANOVA. † $P < 0.001$ when TNF- α compared with saline placebo treatments, as determined by ANOVA. * $P < 0.05$, as determined by post-hoc Student's t test for treatment effect.

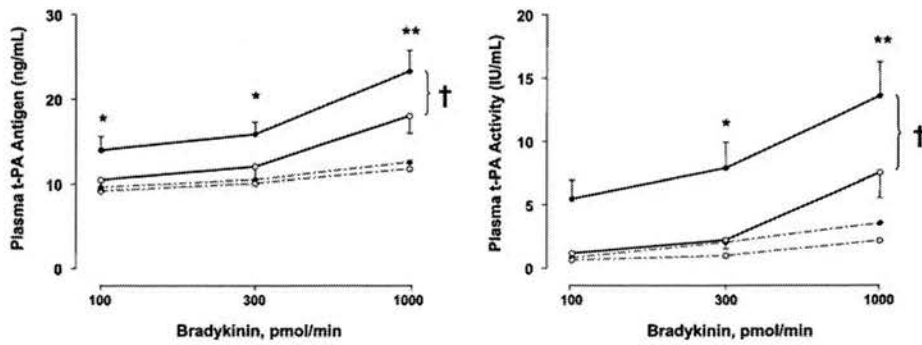


Figure 4 Plasma concentrations of t-PA antigen (left-hand panel) and t-PA activity (right-hand panel) during bradykinin infusion following pretreatment with TNF- α (●) or saline placebo (○)

† $P < 0.001$ when TNF- α compared with saline placebo treatments, as determined by ANOVA. * $P < 0.05$ and ** $P < 0.01$, as determined by post-hoc Student's t test for treatment effect.

release. However, this association may arise from common aetiological factors and does not establish a causal relationship. A recent meta-analysis has suggested that increases in plasma t-PA concentrations largely reflect the presence of concomitant cardiovascular risk factors [7]. We therefore sought to induce vascular inflammation in a representative sample of our study population. Inducing systemic inflammation will have many biological actions and could be confounded by indirect or extra-vascular effects. This may explain some of the differences between our present results and those of previous work in healthy volunteers using *Salmonella typhi* vaccination [9,10]. We chose to employ an acute local inflammatory model [13] to assess the direct effects of intra-arterial TNF- α administration on t-PA release. We were able to induce local vascular inflammation with a rise in local plasma IL-6 concentrations to levels comparable with those seen in patients with unstable angina [17], without evidence of a systemic inflammatory response or change in plasma t-PA or PAI-1 concentrations in the non-infused arm. This local vascular inflammation caused a slow onset and sustained increase in plasma t-PA concentrations that continued for at least 2 h after cessation of the TNF- α infusion. This establishes that vascular inflammation directly causes endothelial t-PA release in humans.

The mechanism of TNF- α -induced t-PA release has not been established. Inflammatory cytokines, such as TNF- α , may cause t-PA release via induction of local thrombus formation, activation of specific cellular receptors or through generation of secondary mediators within the local vasculature. The former seems unlikely given that we observed no increase in prothrombin F1+2, a sensitive marker of *in vivo* thrombin generation [15].

Although it is likely that plasma t-PA concentrations are increased by inducing endothelial injury, smooth muscle cells, macrophages and monocytes also express t-PA mRNA following stimulation by inflammatory cytokines within atherosclerotic plaques [18] and could theoretically contribute to this fibrinolytic response.

Effects of TNF- α on vasomotor function

Impaired vasodilator responses to acetylcholine [19,20] and SNP [20] in patients with cardiovascular risk factors predict an increased risk of adverse cardiovascular events. Previous studies in healthy volunteers have shown acute systemic inflammation is associated with a transient impairment in vasomotor function [9]. In the present study, we have now shown that, in patients with CHD, acute local vascular inflammation decreases the vasomotor response to both acetylcholine and SNP. Acetylcholine is known to stimulate NO (nitric oxide) production via activation of eNOS (endothelial NO synthase) and, together with the impaired SNP response, these findings suggest that local arterial inflammation can decrease NO bioavailability. Interestingly, impaired vasodilator responses to both acetylcholine and SNP have been correlated with plasma TNF- α concentrations in patients with rheumatoid arthritis, a chronic inflammatory condition which is itself associated with an excess cardiovascular risk [21]. Furthermore, intra-arterial infusion of the free radical scavenger vitamin C restores forearm blood flow responses in patients with CHD and elevated serum levels of CRP [22].

Our results suggest that acute inflammation is associated with impaired NO dependent smooth muscle relaxation in response to direct NO donors such as SNP or endogenously derived NO following stimulation of eNOS. Several lines of evidence support the contention that cytokines, such as TNF- α , may impair NO-dependent signalling. TNF- α decreases eNOS expression [23] as well as increasing reactive oxygen species, such as superoxide anion, that rapidly inactivate NO and are directly cytotoxic to vascular tissues. TNF- α also increases plasma ADMA (asymmetric dimethylarginine), an endogenous inhibitor of eNOS that inhibits endothelium-dependent vasodilatation [24]. Finally, inflammatory states may increase iNOS (inducible NO synthase) expression which is associated with receptor uncoupling and endothelial dysfunction [25].

Bradykinin-induced vasodilatation was unaltered by pretreatment with TNF- α . Previous work has suggested that NO contributes only a small proportion (approx. 15%) to bradykinin-induced vasodilatation [26,27] and does not contribute to the mechanism of bradykinin-induced t-PA release [28]. Moreover, in patients with vascular dysfunction, there may be an increased contribution of EDHF (endothelium-derived hyperpolarizing factor) to smooth muscle vasorelaxation [29], particularly if NO is consumed by free radicals generated by locally active inflammatory cells. These observations are consistent with our present findings of impaired acetylcholine- and SNP-induced vasodilatation, but preserved bradykinin responses.

Effects of TNF- α on acute endogenous fibrinolysis

Despite the presence of higher baseline plasma t-PA concentrations in patients with atherosclerosis [7], we have shown in the present study that direct intra-arterial TNF- α infusion increases bradykinin-induced t-PA release in patients with stable CHD. Thus, although vasorelaxation was impaired, acute inflammation initiates a sustained increase in both basal and stimulated t-PA release. The mechanism of this effect is unknown, but may involve direct endothelial injury, up-regulation of t-PA synthesis or alterations in bradykinin receptor expression.

Under some circumstances, increases in t-PA may protect against the propagation of intravascular thrombosis and thereby avoid the development of an acute coronary syndrome. However, elevations in plasma t-PA concentrations may reflect more widespread endothelial dysfunction and a dominant pro-inflammatory vascular response that may overwhelm any locally protective pro-fibrinolytic effect. Indeed, the pro-fibrinolytic actions of vascular inflammation may potentiate degradation of extracellular matrix and aggravate plaque instability [30]. The clinical outcome of acute vascular inflammation may, therefore, depend upon the relative balance between the protective antithrombotic actions and potential plaque destabilization associated with increased vascular t-PA release.

In the present study, all patients who received TNF- α and placebo infusion were already receiving secondary preventative therapy, including aspirin and lipid-lowering medications. Although these may have influenced the vascular response to TNF- α , it was considered unethical to withhold these and, in clinical practice, a large proportion of patients presenting with acute coronary syndromes and raised inflammatory markers are already established on such therapies. As our study design was focused on the question of the link between vascular inflammation and t-PA release in patients with CHD, we did not include a control population of healthy subjects. However, we have shown previously [13] that intra-

arterial TNF- α enhances endothelium-dependent t-PA release by a similar degree in younger healthy volunteers. Although we have again demonstrated that smokers have impaired endothelial responses, including t-PA release [14], intra-arterial TNF- α increased plasma t-PA concentrations to a similar degree in both smokers and non-smokers with established CHD.

We have found that, although TNF- α adversely affects NO-dependent vasodilatation, it enhances other protective mechanisms, such as the endogenous fibrinolytic capacity. This reflects the complex and pleiotropic nature of TNF- α which functions as part of the normal host surveillance mechanisms and response to tissue injury. Although we only determined the effect of acute vascular inflammation in 12 patients, our results may explain some of the contradictory findings of previous clinical studies. For example, in patients with heart failure, TNF- α antagonism causes marked improvements in endothelium-dependent vasodilatation [31], but has failed to demonstrate clinical benefit in randomized controlled trials [32]. Thus the benefits of restoring endothelium-dependent vasomotor function by TNF- α antagonism may be counterbalanced by inhibiting other potentially beneficial acute effects, such as enhancing endogenous t-PA release.

Conclusions

In the present study, we have shown that, in patients with stable CHD, plasma fibrinolytic factors are correlated with CRP, a sensitive and prognostically relevant marker of vascular inflammation. We have also demonstrated that acute vascular inflammation directly impairs vasomotor function whilst enhancing endothelial t-PA release. We suggest that the adverse prognosis associated with elevated plasma t-PA concentrations reflects a causative association with vascular inflammation and injury, rather than representing a marker of occult plaque rupture.

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Phosphodiesterase type 5 inhibition does not reverse endothelial dysfunction in patients with coronary heart disease

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CARDIOVASCULAR MEDICINE

Phosphodiesterase type 5 inhibition does not reverse endothelial dysfunction in patients with coronary heart disease

S D Robinson, C A Ludlam, N A Boon, D E Newby



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Objectives: To investigate whether sildenafil citrate, a selective phosphodiesterase type 5 inhibitor, may improve endothelial vasomotor and fibrinolytic function in patients with coronary heart disease.

Design: Randomised double blind placebo controlled crossover study.

Patients and methods: 16 male patients with coronary heart disease and eight matched healthy men received intravenous sildenafil or placebo. Bilateral forearm blood flow and fibrinolytic parameters were measured by venous occlusion plethysmography and blood sampling in response to intrabrachial infusions of acetylcholine, substance P, sodium nitroprusside, and verapamil.

Main outcome measures: Forearm blood flow and acute release of tissue plasminogen activator.

Results: Mean arterial blood pressure fell during sildenafil infusion from a mean (SEM) of 92 (1) to 82 (1) mm Hg in patients and from 94 (1) to 82 (1) mm Hg in controls ($p < 0.001$ for both). Sildenafil increased endothelium independent vasodilatation with sodium nitroprusside ($p < 0.05$) but did not alter the blood flow response to acetylcholine or verapamil in patients or controls. Substance P caused a dose dependent increase in plasma tissue plasminogen activator antigen concentrations ($p < 0.01$) that was unaffected by sildenafil in either group.

Conclusions: Sildenafil does not improve peripheral endothelium dependent vasomotor or fibrinolytic function in patients with coronary heart disease. Phosphodiesterase type 5 inhibitors are unlikely to reverse the generalised vascular dysfunction seen in patients with coronary heart disease.

The endothelium is important in the regulation of vascular function including local blood flow and endogenous fibrinolysis. Coronary heart disease (CHD) and its risk factors, such as cigarette smoking, hyperlipidaemia, and hypertension,¹⁻³ are associated with impaired endothelium dependent vasorelaxation and reduced endothelial release of the endogenous fibrinolytic factor tissue plasminogen activator (t-PA).⁴⁻⁵ These aspects of endothelial function are important, since plasma fibrinolytic variables and endothelium dependent vasodilatation independently predict future cardiovascular risk.⁶⁻⁷

Nitric oxide is a key factor linked to the beneficial protective effects of the endothelium and a decrease in nitric oxide bioavailability favours atherogenesis.⁸ Nitric oxide exerts many of its biological effects through generation of cyclic guanosine monophosphate (cGMP) after activation of soluble guanylate cyclase. Phosphodiesterase type 5 (PDE5) inactivates cGMP within vascular smooth muscle and thus negatively regulates nitric oxide mediated cellular actions.⁹ Recently, highly selective PDE5 inhibitors that prolong the action of cGMP and thereby enhance nitric oxide mediated effects have become available for clinical use.

It has been suggested that the PDE5 inhibitor sildenafil citrate (Viagra; Pfizer) can improve endothelial vasomotor function in the peripheral circulation of healthy cigarette smokers¹⁰ and the coronary circulation of patients with CHD.¹¹ Although we have previously reported a link between nitric oxide and acute t-PA release,¹² the potential beneficial effects of sildenafil on t-PA and its inhibitor, plasminogen activator inhibitor type 1 (PAI-1), are unknown.

We therefore hypothesised that sildenafil would favourably alter endothelium dependent vasomotor function and acute t-PA release in patients with stable CHD. If so, PDE5 inhibitors may become a useful adjunctive treatment

and confer secondary preventative benefits on patients with CHD.

METHODS

Patients

Sixteen male patients with stable CHD and eight age matched healthy control men participated in the study. The investigation was undertaken with the approval of the local research ethics committee, with the written informed consent of each patient, and in accordance with the principles outlined in the Declaration of Helsinki.

A history of CHD was confirmed by angiographic evidence of $> 50\%$ luminal stenosis of at least one major epicardial coronary vessel or a history of myocardial infarction (confirmed by a serial rise in creatine kinase of twice the upper limit of the normal reference range and the development of pathological Q waves in at least two contiguous leads of the ECG). Nitrate medications were withdrawn for 48 hours before each visit and other medications were withheld on the morning of study. Patient exclusion criteria were significant cardiac failure, renal impairment, systolic blood pressure < 100 or > 190 mm Hg, and diabetes mellitus. Control subjects were healthy normotensive euglycaemic non-smokers without any history of cardiorespiratory or vascular disease and were not taking any regular medications. No participant had received sildenafil or other phosphodiesterase inhibitors before or during participation in this study.

Abbreviations: cGMP, cyclic guanosine monophosphate; CHD, coronary heart disease; FBF, forearm blood flow; PAI-1, plasminogen activator inhibitor type 1; PDE5, phosphodiesterase type 5; t-PA, tissue plasminogen activator

Measurements

Forearm blood flow (FBF) was measured in both forearms by venous occlusion plethysmography with mercury in silastic strain gauges applied to the widest part of the forearm as previously described.¹²⁻¹⁴ During measurement periods the hands were excluded from the circulation by rapid inflation of the wrist cuffs to a pressure of 220 mm Hg with E20 rapid cuff inflators (DE Hokanson Inc, Bellevue, Washington, USA). Upper arm cuffs were inflated intermittently to 40 mm Hg for 10 seconds in every 15 seconds to achieve venous occlusion and obtain plethysmographic recordings. Analogue voltage output from an EC-4 strain gauge plethysmograph (DE Hokanson) was processed by an analogue to digital converter and Chart version 5 software (AD Instruments Ltd, Chalgrove, UK). Instruments were calibrated with the internal standard of the plethysmograph. Blood pressure and heart rate were monitored in the non-infused arm by a semiautomated non-invasive sphygmomanometer (Agilent V24; Phillips Medical Systems). Mean arterial pressure was defined as the diastolic pressure plus a third of the pulse pressure.

Plasma t-PA and PAI-1 antigen concentrations were measured as previously described with enzyme linked immunosorbent assays (Coaliza t-PA and PAI-1; Chromogenix AB, Mölndal, Sweden) at baseline, after sildenafil or placebo, and during intra-arterial substance P.^{4 12 14} Haematocrit was determined by an automated Coulter counter (ACT.8; Beckman-Coulter, High Wycombe, UK). Biochemical assays were undertaken on the fasting venous samples by the hospital clinical laboratory facility.

Study design

Participants were requested to abstain from alcohol for 24 hours and from food, caffeine-containing drinks, and tobacco for at least four hours before each study. All studies were carried out in a quiet temperature controlled room maintained at 22–25°C. Each participant attended at 9 am on two separate occasions at least two weeks apart and received matched placebo and sildenafil in a randomised double blind crossover design.

While participants rested recumbent, strain gauges and cuffs were applied. A 17 gauge venous cannula was inserted into the antecubital vein of each arm and a 23 gauge cannula into the dorsal foot vein for the administration of either intravenous sildenafil or matched placebo. The brachial artery of the non-dominant arm was cannulated with a 27-SWG needle (Cooper's Needle Works Ltd, Birmingham, UK) under local anaesthesia. The intra-arterial infusion rate was maintained constant at 1 ml/min throughout the study with an IVAC syringe pump (Alaris Medical Ltd, Basingstoke, UK).

Saline was infused intra-arterially for the first 20 minutes to allow recording of resting FBF, blood pressure, and heart rate. After this period, sildenafil or matched placebo (Pfizer UK Ltd, Sandwich, Kent, UK) was administered intravenously as a single 26.25 mg bolus over five minutes, then as a continuous infusion of 10 mg/hour to achieve stable plasma concentrations equivalent to the peak concentration of a single 100 mg oral dose (pharmacokinetic data, Pfizer UK Ltd). Twenty minutes after the sildenafil or placebo infusion was started, basal FBF was determined and thereafter acetylcholine (5, 10, and 20 µg/min; Novartis UK Ltd, Farnborough, UK), substance P (2, 4, and 8 pmol/min; Clinalfa AG, Läufelfingen, Switzerland), sodium nitroprusside (2, 4, and 8 µg/min; David Bull Laboratories, Warwick, UK), and verapamil (10, 30, and 100 µg/min; Abbott UK Ltd) were infused intra-arterially for six minutes at each dose. Acetylcholine, substance P, and sodium nitroprusside were given in a random order and separated by 20 minute saline washout periods but, because of its prolonged vasodilator

Table 1 Baseline characteristics

	Patients	Controls
Age (years)	57 (2)	54 (2)
Body mass index (kg/m ²)	27 (1)	27 (1)
Co-morbidity		
Myocardial infarction	10	0
Hypertension	12	0
Diabetes mellitus	0	0
Previous hyperlipidaemia	15	0
Smoker/non-smoker	1/15	0/8
Medications		
Aspirin	16	0
β Adrenergic blocker	13	0
Calcium antagonist	3	0
Long acting nitrate/nicorandil	2	0
ACE inhibitor, AT II antagonist	5	0
Lipid lowering agent	16	0
Serum urea (mmol/l)	5.5 (0.3)	5.1 (0.4)
Serum creatinine (µmol/l)	92 (3)	95 (4)
Glucose (mmol/l)	5.6 (0.2)	5.5 (0.3)
Total cholesterol (mmol/l)	4.2 (0.2)	5.5 (0.2)*
HDL cholesterol (mmol/l)	1.0 (0.0)	1.1 (0.1)
Triglycerides (mmol/l)	1.7 (0.2)	1.2 (0.2)
Placebo visit		
Heart rate (beats/min)	55 (1)	61 (3)*
MAP (mm Hg)	95 (2)	95 (4)
FBF (ml/100 ml/min)		
Infused arm	2.5 (0.2)	2.2 (0.2)
Non-infused arm	2.3 (0.2)	2.1 (0.4)
Sildenafil visit		
Heart rate (beats/min)	55 (1)	65 (3)*
MAP (mm Hg)	95 (2)	91 (3)
FBF (ml/100 ml/min)		
Infused arm	2.5 (0.2)	2.6 (0.3)
Non-infused arm	2.5 (0.2)	2.4 (0.3)

Data are mean (SEM) or number.

*p<0.001 unpaired *t* test, patients v controls.

ACE, angiotensin converting enzyme; AT II, angiotensin II type I receptor; FBF, forearm blood flow; HDL, high density lipoprotein; MAP, mean arterial pressure.

action, verapamil was infused last. The order of the infusions was maintained constant for each participant across both visits.

Statistical analysis

Plethysmographic data were extracted from Chart data files from which the last five linear recording in each measurement period were averaged and FBF was calculated. Estimated net t-PA antigen was defined as the product of the infused forearm plasma flow (based on the haematocrit and the infused FBF) and the concentration difference between the infused ([t-PA]_{inf}) and non-infused ([t-PA]_{Non-inf}) forearms,^{12 14} where estimated net t-PA release = FBF × (1 – haematocrit) × ([t-PA]_{inf}) – ([t-PA]_{Non-inf}).

Data were examined, where appropriate, by analysis of variance with repeated measures and two tailed Student's *t* test by GraphPad Prism (GraphPad Software, San Diego, California, USA). All results are expressed as mean (SEM). Significance was assigned at the 5% level. On the basis of a previous study,¹⁵ this study had an 80% power to detect a 23% change in plasma t-PA concentrations and a 22% difference in FBF in patients with CHD between sildenafil and placebo at the 5% level.

RESULTS

Most patients with CHD had a history of myocardial infarction, hypertension, and hyperlipidaemia (table 1). Reflecting concomitant treatment, mean resting heart rate (55 (1) v 63 (2) beats/min, respectively, *p* < 0.001, unpaired *t* test) and serum total cholesterol concentration (4.2 (0.2) v 5.5 (0.2) mmol/l, *p* < 0.001) were lower in patients with CHD than in controls. Baseline mean arterial pressure,

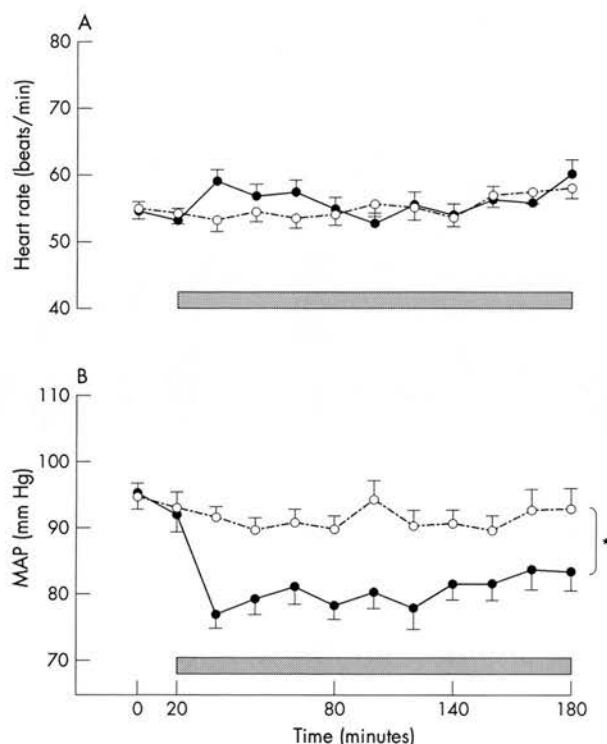


Figure 1 (A) Heart rate and (B) mean arterial pressure (MAP) during sildenafil (closed circles, solid line) or placebo (open circles, dashed line) infusion (shaded box) in patients with coronary heart disease (CHD). * $p < 0.001$ analysis of variance, sildenafil versus matched placebo. Control subject data on file ($p < 0.001$, analysis of variance, MAP sildenafil versus matched placebo).

resting heart rate, baseline FBF, or haematocrit did not differ between the two study visits. Infusions were well tolerated and there were no serious adverse events. For technical reasons, one control subject was unable to complete both visits.

Haemodynamic effects

Over the course of the study, the average mean arterial pressure was lower during sildenafil than placebo infusion in patients with CHD (82 (1) v 92 (1) mm Hg, $p < 0.001$ paired t test sildenafil versus placebo) (fig 1) and control subjects (82 (1) v 94 (1) mm Hg, $p < 0.001$ paired t test). It returned to baseline after discontinuation of infusion (data not shown). Heart rate rose transiently after the sildenafil bolus in both groups (fig 1 and data on file).

Placebo visit

Acetylcholine caused a dose dependent increase in FBF in both groups, although this rise was significantly less in patients with CHD than in controls ($p = 0.005$, analysis of variance) (fig 2). FBF responses did not differ between the two groups during sodium nitroprusside and verapamil infusions (fig 2). There were no significant changes in the non-infused FBF.

Sildenafil and vascular function

Compared with placebo, administration of sildenafil caused no significant difference in the infused FBF during intra-arterial infusion of acetylcholine (at 20 $\mu\text{g}/\text{min}$, mean difference 0.1 ml/100 ml/min, 95% confidence interval (CI) -0.2 to 0.4), substance P (at 8 pmol/min, mean difference 0.5 ml/100 ml/min, 95% CI 0.00 to 0.9), or verapamil (at

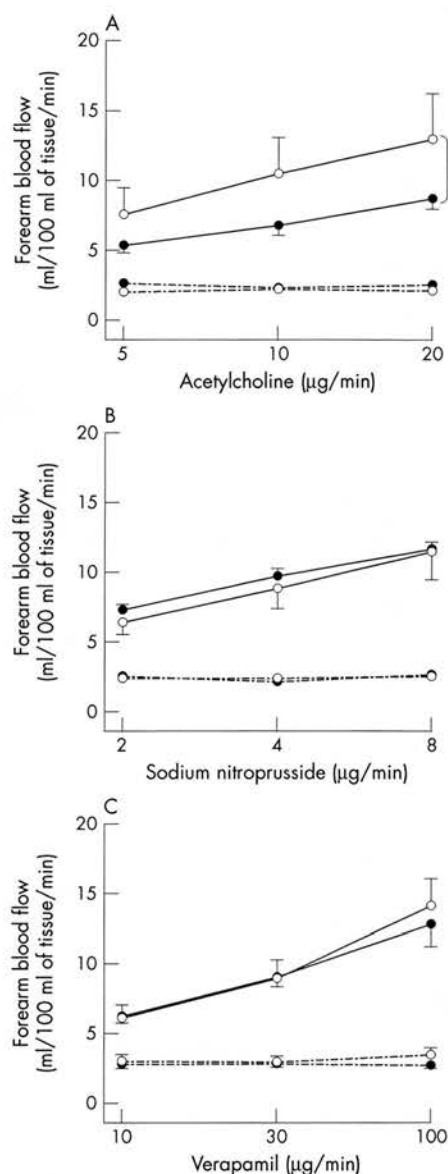


Figure 2 Infused (solid line) and non-infused (dashed line) forearm blood flow in patients with CHD (●) and controls (○) during intrabrachial acetylcholine (panel A), sodium nitroprusside (panel B), and verapamil (panel C) with placebo infusion. $p < 0.001$ analysis of variance, dose response in infused arm; * $p = 0.005$ analysis of variance patients with CHD versus controls.

8 pmol/min, mean difference 0.3 ml/100 ml/min, 95% CI -0.1 to 0.7). However, sildenafil augmented the vasodilatation to sodium nitroprusside in both patients with CHD ($p < 0.05$, analysis of variance) (fig 3) and control subjects ($p < 0.001$, analysis of variance) (fig 4).

Plasma fibrinolytic variables

Baseline plasma t-PA antigen concentrations were unchanged by sildenafil in either group (table 2, fig 5). Substance P caused a dose dependent increase in plasma t-PA concentrations in both patients and controls ($p < 0.01$ for both, analysis of variance) (table 2). The substance P induced increase in plasma t-PA concentrations did not differ during the sildenafil or placebo infusion (at 8 pmol/min, mean difference 0.02 ng/ml, 95% CI -1.15 to 1.18) (table 2) and

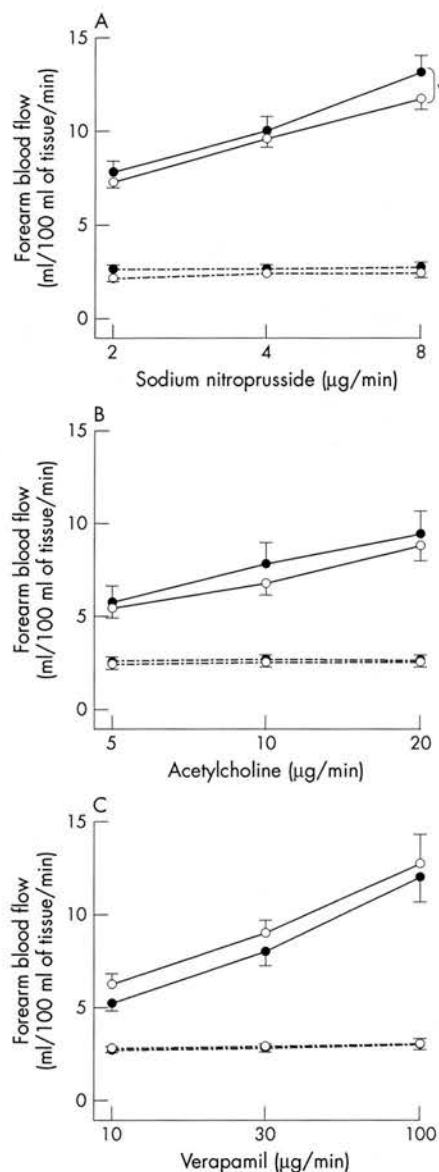


Figure 3 Infused (solid line) and non-infused (dashed line) forearm blood flow in patients with CHD during intrabrachial sodium nitroprusside (panel A), acetylcholine (panel B), and verapamil (panel C) with sildenafil (●) and matched placebo (○) infusion. $p < 0.001$ analysis of variance, dose response in infused arm; * $p < 0.05$ analysis of variance, sildenafil versus matched placebo.

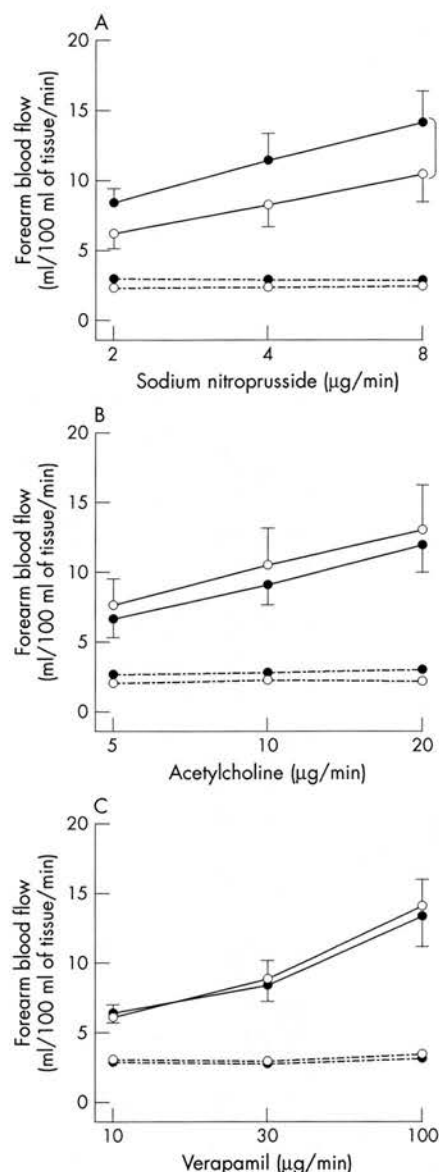


Figure 4 Infused (solid line) and non-infused (dashed line) forearm blood flow in healthy controls during intrabrachial sodium nitroprusside (panel A), acetylcholine (panel B), and verapamil (panel C) with sildenafil (●) and matched placebo (○) infusion. $p \leq 0.01$ analysis of variance, dose response in infused arm; * $p < 0.001$ analysis of variance, sildenafil versus matched placebo.

plasma PAI-1 concentrations did not change significantly throughout either study.

DISCUSSION

We have shown that sildenafil, a selective PDE5 inhibitor, does not modify endothelium dependent vasodilatation or acute t-PA release in men with stable CHD. However, sildenafil did augment the vasodilator effect of the exogenous nitric oxide donor sodium nitroprusside. Thus, while our study confirms the well described interaction of sildenafil with nitric oxide donors,^{10 16} we have found no evidence to support the contention that PDE5 inhibitors improve endothelium dependent vasomotor or fibrinolytic function in patients with CHD.

Compared with matched controls, patients with CHD exhibited impaired endothelium dependent responses to

acetylcholine while having preserved vasodilator responses to the endothelium independent agonists sodium nitroprusside and verapamil. This prognostically significant impairment^{7 17} was evident in patients who were already receiving standard antianginal, antiplatelet, and lipid lowering treatments.

Sildenafil had no effect on peak flow mediated dilatation of the brachial artery in patients with CHD¹¹ and reports on the vasomotor responses of the coronary vessels to sildenafil are conflicting. Herrmann *et al*¹⁸ found no change in coronary artery diameter, blood flow, or coronary vascular resistance, whereas Halcox *et al*¹¹ reported enhanced coronary artery vasodilatation to acetylcholine. Unlike previous studies, we used a more robust double blind randomised placebo controlled crossover study design and have shown that PDE5 inhibition does not alter either endothelium dependent vasomotor or fibrinolytic function in patients with

Table 2 Plasma tissue plasminogen activator (t-PA) and plasminogen activator inhibitor type 1 (PAI-1) concentrations at baseline and during sildenafil and matched placebo infusion in patients with coronary heart disease

	0	Bolus	Continuous infusion (10 mg/h)		
Sildenafil/placebo Substance P dose (pmol/min)			2	4	8
Placebo					
Plasma t-PA antigen (ng/ml)					
Infused arm	9.0 (0.7)	8.7 (0.6)	9.3 (0.6)	9.8 (0.7)	10.5 (0.8)*
Non-infused arm	9.0 (0.6)	9.1 (0.6)	9.1 (0.6)	8.9 (0.6)	9.1 (0.6)
Plasma PAI-1 antigen (ng/ml)					
Infused arm	46.8 (6.9)	43.1 (6.0)	0	0	34.4 (5.4)
Non-infused arm	45.2 (7.1)	45.5 (7.6)	0	0	36.4 (6.3)
Sildenafil					
Plasma t-PA antigen (ng/ml)					
Infused arm	9.0 (0.6)	8.7 (0.6)	9.1 (0.6)	9.4 (0.6)	10.5 (0.7)*
Non-infused arm	9.1 (0.6)	8.8 (0.6)	9.0 (0.6)	8.9 (0.6)	9.0 (0.6)
Plasma PAI-1 antigen (ng/ml)					
Infused arm	44.0 (5.6)	39.6 (4.6)	0	0	36.0 (3.8)
Non-infused arm	47.9 (5.6)	43.2 (4.9)	0	0	38.6 (4.2)

* $p < 0.001$, analysis of variance for t-PA response.Control subject data on file ($p = 0.003$, analysis of variance for t-PA response).

CHD or in age matched controls. Moreover, we used a bolus and continuous intravenous sildenafil infusion to minimise variations in plasma concentrations during the administration of each of the intra-arterial vasodilators. This

is an important study consideration given the short half life of sildenafil in humans.

We observed a decrease in mean arterial pressure in both patients and controls during administration of sildenafil that presumably reflected an augmentation of the vascular effects of basal vascular nitric oxide release and is mediated through an increase in cGMP. Our findings are consistent with the published haemodynamic data from both healthy volunteers¹⁹ and patients with CHD^{18–20} and confirm that we achieved a physiological effect with sildenafil infusion. The consistent vasodilatory response to the nitric oxide independent agonist verapamil makes it unlikely that administration of the PDE5 inhibitor impaired vascular smooth muscle function or obscured potentially beneficial effects on endothelial function. Moreover, both acetylcholine and substance P produced similar, consistent, and reproducible responses on both study days. This suggests that prolonging cGMP actions in patients with established atherosclerosis would not reverse endothelial dysfunction. As would be predicted from its mechanism of action, sildenafil augmented the responses to sodium nitroprusside, an exogenous nitric oxide donor, in both controls and patients with CHD.

There are several potential reasons for the differences observed in the effect of sildenafil on the acetylcholine and sodium nitroprusside responses. The modest decrease in acetylcholine induced vasodilatation^{21–22} seen after nitric oxide synthase inhibition suggests that non-nitric oxide dependent pathways such as endothelium derived hyperpolarising factor (EDHF) may predominate particularly in the presence of endothelial dysfunction.^{23–24} Furthermore, differences in the relative contribution of endothelium derived nitric oxide across vascular beds may explain some of the previously conflicting data on the vascular responses to sildenafil.^{11–25–26} As well as endothelial dysfunction, atherosclerosis is associated with high concentrations of free radicals such as superoxide anion that rapidly react with nitric oxide to generate peroxynitrite, a powerful oxidant species that induces significant cellular damage and directly inhibits soluble guanylate cyclase.²⁷ Elegant studies in animals with specific knockouts of nitric oxide dependent pathways also suggest that tissue specific downregulation of nitric oxide/cGMP, including cGMP dependent protein kinase, may be an early feature of endothelial dysfunction in atherosclerotic conditions.^{28–29} PDE5 inhibition would not be anticipated to influence changes in oxidative stress or directly affect cGMP independent nitric oxide molecular targets that contribute to endothelial dysfunction and

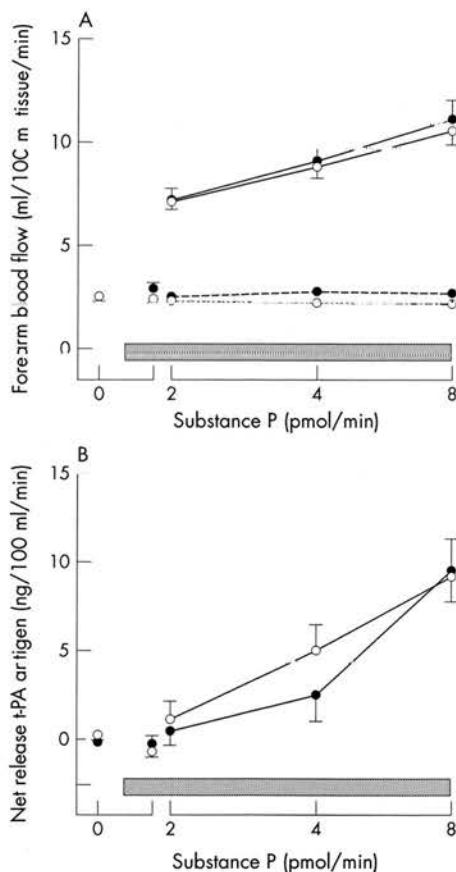


Figure 5 Infused (solid line) and non-infused (dashed line) forearm blood flow (panel A) and estimated net release of tissue plasminogen activator (t-PA) antigen (panel B) at baseline, during sildenafil (●) and matched placebo (○) infusion (shaded box), and subsequently with intrabrachial substance P (2, 4, 8 pmol/min) in patients with CHD. $p < 0.01$ analysis of variance for all, dose response for infused arm FBF and net t-PA release.

atherogenesis. Therefore, the contrasting effects of sildenafil on acetylcholine and sodium nitroprusside induced vasodilatation are likely to reflect a major dependence on non-nitric oxide mediated pathways, increased oxidative stress, and decreased nitric oxide bioavailability associated with CHD.

Although the precise mechanism underlying acute t-PA release remains uncertain, several reports have previously suggested involvement of nitric oxide and cyclic nucleotides regulated by phosphodiesterases. In animals, pentoxifylline and its analogues, non-selective phosphodiesterase inhibitors, increased acute t-PA release³⁰ and potentiated the effects of thrombolytic treatment.³¹ We and others have reported acute endothelial t-PA release during intra-arterial substance P,^{4,14} bradykinin,³² and methacholine³³ infusions, as well as an inverse relation between acute t-PA release and atherosclerotic plaque burden within the coronary circulation.³⁴ In the present study, we have again shown a rise in both plasma t-PA antigen concentrations and net t-PA release with local intra-arterial substance P infusion. However, infusion of sildenafil did not change basal plasma t-PA concentrations or substance P induced t-PA release. Therefore, enhancement of cGMP apparently does not directly augment endothelial t-PA release in humans.

Although we found no such change in acute t-PA release with substance P, sildenafil may improve the response to other agonists such as bradykinin, which causes B2 receptor mediated prostacyclin and nitric oxide generation.³⁵ However, the dominant mechanism of t-PA release with bradykinin appears to be nitric oxide and prostacyclin independent with coupling of the B2 receptor to the calcium dependent G_q/phospholipase C-beta pathway.³⁶ Some of the conflicting results on the effect of nitric oxide synthase inhibitors on t-PA release may suggest that there is physiological redundancy within the nitric oxide dependent pathways which contribute to the regulation of acute t-PA release in humans.

Study limitations

In light of the haemodynamic changes seen in our study, intrabrachial infusion of sildenafil in subsystemic locally active doses would be one approach to assess the direct vascular actions of PDE5 inhibition. However, sildenafil is metabolised by the liver to an active metabolite that accounts for nearly half of its phosphodiesterase inhibitory activity. Local intra-arterial infusion would not assess the action of this important metabolite. The effect of long term PDE5 inhibitor therapy remains unclear, and further studies with women and patients with diabetes mellitus would be of interest, as these groups may show differences within nitric oxide dependent pathways.^{37,38}

Conclusion

Despite being highly effective in the management of erectile dysfunction, sildenafil does not modify endothelium dependent vasomotor or fibrinolytic function in patients with CHD. Phosphodiesterase inhibitors have already shown promise as novel treatments for conditions such as chronic heart failure²⁵ and pulmonary hypertension,³⁹ and these areas clearly warrant further research. However, on the basis of our results, we believe that PDE5 inhibitors are unlikely to reverse the generalised vascular dysfunction seen in patients with CHD.

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Competing interests: SDR has received financial support for attending scientific meetings from Pfizer Ltd; NAB was a member of a drug advisory committee evaluating sildenafil and has received and supervised research grants from Pfizer Ltd; DEN holds unrestricted educational grant awards and has undertaken paid consultancy for Pfizer Ltd.

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IMAGES IN CARDIOLOGY

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Pseudo-supraventricular tachycardia

A 79 year old man with dilated phase of hypertrophic cardiomyopathy and a history of antero-septal myocardial infarction was admitted because of frequent episodes of tachyarrhythmia requiring cardioversion. A 12 lead ECG demonstrated small positive waves (closed circles) followed by larger positive waves (open circles) in limb leads and leads V4–6, which, at first glance, seemed to represent P waves followed by QRS complexes (panel A). A closer look indicated that the presumed P waves (closed circle) in leads V4–6 corresponded to R waves (arrow-head) in leads V1 and V2 as well as dissociation of true P waves (arrows) from QRS complexes. The diagnosis of ventricular tachycardia was confirmed by electrophysiology study, which revealed that the first and second positive wave corresponded to the local ventricular potential recorded from the apex of right and left ventricle, respectively (panel B). Catheter ablation of the ventricular tachycardia was not possible because the tachycardia was not stable. The patient was treated with mexiletine and followed uneventfully.

This case re-emphasises the importance of recording a 12 lead ECG for the diagnosis of arrhythmia, even when the diagnosis from an ECG with fewer leads such as telemetry seems to be straightforward (sinus tachycardia or supraventricular tachycardia in this case).

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